Relationship between Serum Lipoproteins and Sex- and Adrenal Cortical Hormones in Men

by

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Abstract

This study examined the relationships between sex hormones (serum concentrations and urinary excretion of testosterone and estradiol) and serum lipid, lipoprotein-lipid and apolipoprotein concentrations in healthy Chinese men. The results showed a number of statistically significant associations: The urinary unconjugated estradiol excretion was directly, but weakly, associated with serum concentrations of HDL_3-C ($r=0.3073$, $p=0.038$). When expressed as a percentage of the total estradiol excretion this association increased in strength ($r=0.401$, $p=0.0058$). Total urinary estradiol excretion was weakly and inversely associated with serum total cholesterol concentration ($r=-0.3102$, $p=0.0359$), and LDL-C ($r=-0.307$, $p=0.0379$) and more strongly with apolipoprotein B ($r=-0.379$, $p=0.0094$). However, neither urinary unconjugated nor total testosterone excretions were associated with any lipoprotein-lipids in serum univariately. Similarly urinary free cortisol showed no significant associations with any lipoprotein variable.

On stepwise multiple regression which included all the sex hormone variables the association between unconjugated estradiol and HDL_3-C became stronger ($t=2.8$, $p=0.0076$) and the association with apolipoprotein B became significant ($t=2.32$, $p=0.025$). Similarly the associations between total estradiol excretion and total cholesterol, LDL-C, and apolipoprotein B became significantly stronger ($t=-2.6$, $p=0.0126$; $t=-2.53$, $p=0.0153$; $t=-3.06$, $p=0.0038$, respectively), and a weak association with HDL_3-C developed ($t=-2.05$, $p=0.0468$).

When triglyceride concentration was included in the model (the metabolism of triglyceride is known to be closely linked with that of high density lipoprotein and its subfractions) the following associations were found: Urinary estradiol was not associated with HDL-C and HDL_2-C, but was with HDL_3-C. However, HDL-C and HDL_2-C remained directly associated with serum testosterone (total and/or unbound) in

this model. ApoA-I, independently of triglyceride, correlated directly and significantly ($p = 0.02$) with SHBG after multiple linear regression with all possible confounding variables.

The results of this study are consistent with the observation that pre-menopausal women have lower serum concentrations of the atherogenic lipoproteins LDL-C and apoB. Pre-menopausal women are at lower risk for the development of coronary artery disease. However other reports have suggested that men with CAD actually have higher estrogen levels than men without disease, implying that any beneficial effect on lipoproteins in men from higher estrogen levels provides no protection. The existence of correlations is not proof of metabolic cause and effect. Further studies in this area should be pursued because this might be helpful in the elucidation of the relation between sex hormone secretion in normal and abnormal lipid status.

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Chapter I. Introduction

Based on a single biological sample, the associations between serum sex hormone and lipoprotein concentrations have shown many discrepancies (Heller, *et al.* 1983; Semmens *et al.,* 1983; Stefanick *et al.,* 1987; Kiel, *et al.* 1989). Since the plasma hormone levels reflect episodic and diurnal variations, associations may be obscured. In this study an attempt was made to reduce such variation by measuring serum concentrations on two occasions about 2-3 weeks apart. Furthermore, on the basis that the 24-hr urinary excretion of sex hormones is likely to be independent of short term fluctuations in plasma hormone levels and that no study to date has examined the relationship of the concentrations of serum lipoprotein-lipids to the excretion of urinary sex hormones, urinary sex hormone excretion in addition to analysis of sex hormones in plasma was determined. In this project, sampling of blood and 24-hr urine collections on two different occasions, 2-3 weeks apart, were used to estimate and account for the biological variation. In addition, to reduce another source of variation, biochemical assays were done in duplicate because analytical variation as well as intra-individual variation will contribute to the overall variation.

Four considerations need to be noted:

1. Urinary conjugated testosterone in men is not uniquely a metabolite of plasma secreted testosterone. This is based on the finding of Camacho & Migeon (1964) and Korenman and Lipsett (1964), who showed a substantial amount of adrenal androgens (mainly androstenedione) are transformed, metabolized and excreted as testosterone glucuronide in urine.

2. Urinary un-conjugated (extractable) testosterone in men mostly originates from gonadal secretion rather than from exogenous testosterone or from peripheral transformation. This is based on the finding of Camacho & Migeon (1964) that after the injection of radiolabelled testosterone and other closely related steroids

(such as 17-α hydroxyprogesterone, androstenedione and DHEA) into normal men only a very insignificant amount (from 0 to 0.007%) of the injected dose of radiolabelled testosterone is recovered in the ether extracted fraction of crude urine, while the conjugated fractions of the testosterone account for practically all of the radioactivity recovered.

3. In animal studies measurements of urinary un-conjugated steroids are reliable indices of the secretory activity of the adrenal-gonadal axis after hCG administration (Fenske, 1988; 1989).

4. Most of the plasma testosteone in men is secreted by the gonads (Lipsett ***et al.,*** 1968).

In 1980, Masarei's group in Australia was one of the first to ask the question "to what extent does variation in endogenous sex hormone levels contribute to variation in serum lipoprotein concentration?" They documented that plasma estradiol concentrations show a weak positive correlation with HDL-C, while plasma sex hormone-binding globulin (SHBG) levels are strongly correlated with HDL-C in both post- and pre-menopausal women. In 1983, subsequent studies performed by the same group showed a direct association between total testosterone and HDL-C but this became an inverse association on controlling for SHBG suggesting that there is an *inverse* correlation between unbound endogenous testosterone and HDL-C. They showed a *direct* correlation between SHBG and HDL-C in men and women. The work of Goldberg and his co-workers (1985) supported the findings of Masarei's group by *suppression* of plasma testosterone by a long-acting gonadotropin releasing hormone analog ($LHRH_A$) in normal men, which leads to an *increase* in serum total and high density lipoprotein cholesterol and apolipoprotein A-I and B. Stefanick and co-workers (1987) showed that the plasma total testosterone levels had significant *inverse* associations with HDL cholesterol and HDL_2 mass concentrations, and a

significant negative relationship was found between SHBG and triglycerides.

Nevertheless, the data of other workers pursuing this subject showed a different result by suggesting a *direct* relationship of HDL-C and unbound testosterone in men. Among these, the work of Heller and his co-workers (1983) , which appeared almost at the same time as the paper by Masarei's group (Semmens ***et al.,*** 1983) is a good example, although salivary testosterone was used as an index of free hormone levels. Kiel ***et al.*** (1989) presented a *negative* result showing that there is *no association* between total- or free-testosterone and HDL-C, whereas there is a consistently *positive* correlation between total estradiol or calculated free estradiol and both total cholesterol and HDL-C. In addition, they documented a complex interaction between endogenous testosterone and estradiol in relation to the lipoprotein profile. The above results, though not consistent, all suggest that sex hormones exert an important effect on lipoprotein metabolism and play a key role in defining the lipoprotein profile in men.

In view of the controversial results of the previous studies, it was decided to re-assess the problem by studying not only serum sex hormone levels and the concentrations of lipoprotein-lipids and apolipoproteins in human male subjects (not in women because of the additional problem of cyclical changes) but also the urinary excretion rate as a reflection of production and secretion of sex hormones.

Nanjee and Miller (1989) reported that plasma cortisol level, independent of plasma level of triglyceride, HDL-C and androstenedione, is positively correlated with plasma low density lipoprotein cholesterol (LDL-C) concentration in men. As there is considerably diurnal and time-to-time variation in plasmal cortisol levels, which would turn to obscure any association, it is interesting to know how cortisol production and LDL-C might be correlated.

Objectives

The aims of this project were to investigate the relationship between the concentrations of serum lipoproteins and the excretion of the sex hormones in normal men in order to answer the following questions: (1) Do endogenous sex hormones contribute to the variation in serum lipoprotein-lipids? (2) To what extent do sex hormones affect the plasma lipoprotein-lipids? (3) If the excretion of urinary sex hormone reflects the secretion rate and the production rate of the endogenous sex hormones, do urinary sex hormone excretions account better for variation in plasma lipoprotein-lipids than serum sex hormone concentrations? (4) Will the urinary free cortisol which reflects its production rate explain more clearly in its association with serum lipoproteins?

Chapter II. Literature Review

II.1. Lipoprotein-Lipids

II.1.1. General Concept and Metabolism of Lipoprotein-lipids

All lipids from dietary sources or body synthesis, except non-esterified fatty acid (NEFA) which is bound by albumin, are transported in plasma in the form of lipoproteins. The spherical particles of lipoproteins of varying sizes have unique flotation rates (sf), hydrated densities (d), and electrophoretic mobilities. Based on the physical properties of plasma lipoproteins, six distinct classes of lipoproteins can be isolated from postprandial plasma, namely chylomicrons (d <0.95 g/ml), VLDL (very-low-density lipoproteins, d <1.006 g/ml), IDL (intermediate-density lipoproteins, d =1.006-1.019 g/ml), LDL (low-density lipoproteins, d =1.019-1.063 g/ml), HDL (high-density lipoproteins, d =1.063-1.21g/ml) and Lp(a) [lipoprotein(a),d=1.051-1.082g/ml]. Apolipoproteins are the protein fractions of the lipoprotein particles that react with a series of enzymes and/or tissue receptors, and are responsible for further metabolism of the particles. A lipoprotein molecule consists of an outer monolayer of apolipoprotein and polar lipids (phospholipid and free cholesterol) and an inner core of neutral lipids (triglyceride and esterified cholesterol) which is non-polar (Tietz[2], 1986). Lipoproteins also differ importantly in terms of their content of apolipoproteins. For example, apoA is mainly associated with HDL, apoB with LDL, apoE with VLDL and VLDL- and chyromicron remnants, apoC with chylomicrons and VLDL, and apo(a) with Lp(a). There are heterogeneities of apolipoproteins which may have structural, metabolic and/or genetic significance of the lipoproteins, such as apoA-I and apoA-II for apoA; ApoB48 and apoB100 for apoB; the subunits of apo(a) for Lp(a). ApoA-I plays a central role in determination of the structure and plasma concentration of HDL, and apoA-II in determination of the HDL size (Funke ***et al.***, 1991; Schultz ***et al.***,

1992; Cheung *et al.*, 1993). ApoB100 is the ligand for the LDL receptor, but apoB48 which is associated with chylomicron does not bind with it (Thompson, 1990). The apo(a) isoforms, associated with high Lp(a) plasma concentrations, may represent a stable genetic trait in subjects with CHD in different populations (Sandholzer[2] *et. al.*, 1992). In the apoE4 phenotype subjects tend to have higher level of total cholesterol in the body whereas in apoE2 homozygous phenotypes higher level of VLDL cholesterol (Mahley and Innerarity, 1983; Kesäniemi *et al.*, 1987; Mahley, 1988). It is known that subjects with apoE2 homozygous phenotypes, in combination with a second independent metabolic disorder, develop Type III hyperlipoproteinemia (Feussner *et al.*, 1990). (For review, see Bhatnagar and Durrington, 1991).

Most of the cholesterol in the plasma of fasting normal subjects is carried by LDL-C, the rest being found in VLDL-C, and HDL-C. Most of the endogenous triglyceride is carried by VLDL. Thus, measurement of the total cholesterol and triglyceride in the fasting state plasma (usually after a 12-hour fast) gives the sum of the contributions from each lipoprotein class. Under normal circumstances there are virtually no IDL-C or chylomicrons found in fasting plasma. However, raised IDL-C or chylomicrons remnants, as in type III hyperlipoproteinemia, are thought to increase the risk of atheromatous disease because the macrophages and other cells of the reticuloendothelial system take up these remnants to form foam cells in the arterial wall. Similar mechanisms also take place for LDL particles modified by acetylation or peroxidation *in vivo*. Thus high plasma levels of LDL-C show a marked direct association with atheroma formation (Fuster *et al.*, 1992). An alternative means of quantitating LDL-C is to measure apoB in LDL-C. Because there is one apoB molecule to each LDL particle, apoB provides a better index of the number of LDL particles present in plasma (Levinson and Wagner, 1992).

LDL is the major extracellular cholesterol source for cells. All human cells obtain and degrade LDL through the LDL-receptor pathway. The LDL receptor is a transmembrane glycoprotein on the surface of most cells in the body. It has high binding affinity for the apoB molecule in an LDL particle. Once LDL is internalized, it is transferred into lysosomes and digested by lysosomal enzymes. The cholesteryl ester in the central core of the LDL particle is hydrolyzed and free cholesterol is released and becomes available for steroidogenesis (in liver, gonads, adrenal cortex and placenta), for formation of lipoproteins, and for conversion into bile acid for excretion by the liver (Brown and Goldstein, 1986). ApoE also can be recognized by LDL (B, E) receptors on liver cells and is responsible for the removal of the remnants produced by the action of lipoprotein lipase on VLDL. Therefore, decreased activity of hepatic LDL receptors will result in a fall in the rate of VLDL remnant removal from the circulation. Other receptors are also present in human cells, such as the apoE receptor, which only recognizes lipoproteins containing apoE, but does not recognize LDL. Receptors recognizing apoE on chylomicron remnants mediate their rapid removal from the circulation after a fatty meal (Mahley and Innerarity, 1983; Mahley, 1988).

Fatty acids are stored in adipose tissue as triglyceride. The mobilization of triglyceride is controlled by a hormone-sensitive lipase, the activity of which is enhanced by hormones such as noradrenaline and the glucocorticords and inhibited by insulin. Lipolysis results in the release of non-esterified fatty acid (NEFA) and glycerol into plasma. NEFAs are carried by albumin to the liver, heart and skeletal muscle where oxidation of fatty acid is carried out. NEFAs are also taken up by the liver and are re-esterified to triglycerides and phospholipids. Phospholipids are the important components of all cell membranes due to their amphipathic nature, by which the membrane is able to interact with

non-polar (lipoid) and polar (aqueous) environments. Changes of phospholipid composition in the cell membrane may cause membrane damage (Borkman ***et al.,*** 1993). For instance, the immune-induced hydrolysis of (glomerular epithelial cell) membrane phospholipid might account for the development of glomerular membranous nephritis as in the case of *passive Heymann nephritis* (Weise ***et al.,*** 1993).

Besides being an essential component of the membranes of all human cells, the myelin sheaths of nerves, and the outer shell of plasma lipoprotein particles, cholesterol also acts as the precursor of bile acids and steroid hormones which are the major routes of elimination of the synthesized and dietary cholesterol from the body. Bile acids are the major metabolites of cholesterol in the liver, with the rate-limiting enzyme being cholesterol 7-α-hydroxylase which is negatively fed back by bile acids, the end product. The biosynthesis of cholesterol takes place in all human cells. It begins with transformation of acetic acid to acetyl CoA, and then to the 6-carbon compound, mevalonic acid. Six mevalonic acid molecules condense to form squalene, which is then hydroxylated and converted to a 27-carbon skeleton of cholesterol. The rate-limiting enzyme is β-hydroxy-β-methylglutaryl-CoA (HMGCoA) reductase which is important in the formation of mevalonic acid from the reduction of HMG-CoA. HMGCoA reductase is subject to negative feed-back by its end product, cholesterol.

During metabolism of living cells, the esterification of free cholesterol is enhanced by an enzyme ACAT (acyl-CoA-cholesterol acyltransferase) which transfers the fatty acid residue of acyl-CoA to cholesterol. In plasma, lipolysis of chylomicrons and VLDL by lipoprotein lipase (LPL) results in the release of triglycerides, free cholesterol, phospholipids, and apolipoproteins, leaving the hydrolyzed particles (remnants). The remnants acquire apoE from other lipoproteins, which serves as the ligand for LDL receptor present in the liver for

clearance and catabolism of remnants. The released particles are then transferred to the small, dense HDL_3 particle where similar esterification of free cholesterol is also enhanced by an enzyme LCAT (lecithin-cholesterol acyltransferase) to form HDL_2 by transferring fatty acid residues from phospholipids (lecithins) to free cholesterol. This process is associated with reverse cholesterol transport through HDL (Witztum and Schonfeld, 1979; Myant, 1991). The cholesteryl ester of the derived HDL particles that acquire apoE (HDL_2), on the other hand, can also be exchanged from HDL_2-C into VLDL or LDL by cholesteryl ester transfer protein (CETP). CETP is a hydrophobic glycoprotein which binds to triglyceride-rich particles and circulates in a complex with LCAT. This CETP-mediated cholesterol exchange into the apoB-containing lipoprotein (LDL or VLDL) will facilitate the removal of cholesterol when VLDL and LDL particles are taken up by LDL receptors. CETP is associated with the regulation of HDL cholesterol, since by transferring the cholesteryl esters from HDL_2-C via CETP, HDL_2 particles are redigested by hepatic lipase to generate HDL_3. The complex interactions of lipoprotein lipase activity, hepatic lipase activity and fasting HDL_2 levels determine the postprandial triglyceride levels (Silverman ***et al.,*** 1993). The reverse transport of cholesterol by HDL plays a key role for cholesterol removal from the body, and thus in the function of regression of atherosclerosis (Badimon ***et al.***, 1992).

The studies on the role of plasma triglyceride levels as a risk factor for coronary heart disease have been controversial (Barbir ***et al.,*** 1988; Austin, 1989, Criqui ***et al.,*** 1993). However, the four well-known American studies [Framingham Heart Study (FHS), Lipid Research Clinics Prevalence Mortality Follow-up Study (LRCF), Coronary Primary Prevention Trial (CPPT), and Multiple Risk Factor Intervention Trial (MRFIT)] and the British Regional Heart Study (BRHS) have shown a consistent inverse correlation of HDL-C levels and

coronary heart disease mortality rate when differences in analytic methodology were eliminated (Gordon ***et al.,*** 1989). The predictic value of HDL-C for the overall mortality rates of CVD and/or CAD has been proved better than that of the sole measurement of total cholesterol and/or LDL cholesterol (Kannel and Wilson, 1992). In addition to HDL-C, other work has shown that levels of HDL_2-C, a subfraction of HDL-C, are closely inversely related to cardiac vascular disease as in angiographic studies (Miller, ***et al.,*** 1981; Johansson ***et al.,*** 1991; Drexel ***et al.,*** 1992).

Any defects or changes in the rate-limiting enzymes (HMGCoA reductase, cholesterol 7-α hydroxylase, LCAT, ACAT, LPL), and/or in receptors (e.g. apoB or apoE receptors) and CETP by intrinsic or extrinsic factors may result in abnormalities and/or changes of lipoprotein-lipid levels in plasma. Treatment of hyperlipidaemias with drugs is based on the principles of increasing the clearance of apoB-containing lipoproteins by increasing LDL-receptor activity, on lowering hepatic cholesterol synthesis or draining cholesterol with bile acids by sequestration in the distal small gut with resins. Other drugs may work by reducing VLDL production, such as nicotinic acid. (Kannel and Wilson, 1992; Scott, 1993).

Lp(a) is a unique lipoprotein with a density between those of LDL-C and HDL-C. There are resemblances of structural and biochemical properties of Lp(a) and LDL-C. Lp(a) is not a metabolic product of VLDL-C, LDL-C, or chylomicrons, and Lp(a) is not converted to other lipoproteins. It has an independent synthetic pathway (Krempler ***et al.,*** 1980; Houlston and Friedl, 1988). Apo(a) which is the protein moiety of Lp(a), links to apoB-100 by a disulfide bridge and serves as distinctive marker for Lp(a) levels in plasma. The size of apo(a) isoforms reflected in Lp(a) concentrations, is an independent risk factor for predicting the development of coronary heart disease, and is controlled

by the apo(a) gene. There is an inverse relationship between apo(a) size and plasma Lp(a) level (Sandholzer[2] ***et al.,*** 1992). A person's Lp(a) is genetically controlled, independent of age and sex and the plasma concentration of Lp(a) is uniquely stable throughout the whole life span (Lawn, 1992). However, the apo(a) gene only accounts for 40% of the variation in Lp(a) levels, and other factors must contribute (Utermann ***et al.,*** 1989; Scanu and Fless, 1990). Among these, cyclosporin (Webb ***et al.,*** 1993), nicotinic acid (Gurakar ***et al.,*** 1985; Carlson ***et al.,*** 1989; Schmidt ***et al.,*** 1993), fish oil (Scanu and Fless, 1990; Fu ***et al.,*** 1991), and androgens, estrogens, and growth hormone (Farish ***et al.,*** 1991; Henriksson ***et al.***, 1992; Edén ***et al.***, 1993), as well as pregnancy (Panteghini and Pagani, 1991) are good examples. Nevertheless, a more recent paper has suggested that the apo(a) gene is responsible for 91% of the variance of plasma Lp(a) concentration (Boerwinkle ***et al.,*** 1992).

II.1.2. Factors affecting plasma lipoprotein-lipids

Intrinsic (genetic) and extrinsic (environmental) factors both affect the plasma levels of lipoprotein-lipids. Sometimes it is difficult to differentiate these two factors, because they interact and the combined effects may be difficult to predict.

II.1.2.1. Ageing

It has been well documented that age is an important source of variation of plasma lipoprotein-lipid concentrations in both men and women. There is an age-related increase of total cholesterol and LDL-C values in men, and in women not taking sex hormones, between the ages 20-50 years, while the change of HDL-C with age is less prominent and the level remains rather stable at this age range in both sexes. In males, triglyceride and VLDL-C increase with age with a parabolic relationship (an acme is reached between the ages 40-44), while in females a curvilinear increase with age is observed. (Heiss ***et al.***, 1980). Ageing

is also frequently accompanied by an increase in body weight, a decrease in physical activity and changes in hormonal levels, as in the case of postmenopausal women (Thompson, 1990; Stevenson ***et al.,*** 1993). In men, there is an age-related decline of serum total and bioavailable testosterone (Dai ***et al.,*** 1981; Nahoul and Roger, 1990).

II.1.2.2. Obesity

Adiposity is an important source of variation in plasma lipoprotein-lipids levels. An increase in the levels of triglyceride and VLDL-C is found in many obese individuals, although there is little relationship between total cholesterol and obesity (Bray, 1989). However, it is well known that there is an inverse relationship between HDL-C and obesity (Stunkard ***et al.***, 1981; Stubbe ***et al.,*** 1983; Bray, 1989). Furthermore, adiposity has effects on sex-hormone levels and SHBG concentrations. In terms of *body mass index* (BMI) (expressed by the ratio of body weight in kilograms and the square of height in meters) (kg/m^2) adiposity affects the physiological hormonal levels in body. In men, androgens decrease (Zumoff ***et al.,*** 1990) and estrogens increase with obesity (Segal ***et al.,*** 1987). The reduction in total testosterone is usually accompanied by a reduction in the level of SHBG and the fall in SHBG is associated with a rise in the concentration of estradiol and estrogen in the plasma in moderately obese men (Bray, 1989). In morbidly obese men, higher estrogen and lower androgen and sex hormone binding globulin concentrations than those in normal-weight subjects are observed (Segal ***et al.***, 1987; Pasquali ***et al.,*** 1988). In women, the secretion of estrogen decreases on reaching the menopause, with increased adiposity (Kuller ***et al.,*** 1990; Stevenson **et al.,** 1993). The body composition-related sex steroid alterations may affect the lipoprotein-lipids profile (Segal ***et al.,*** 1987). The body fat distribution in terms of *waist and hip ratio* (WHR) (Terry ***et al.,*** 1989), *abdomen-hip ratio* (AHR) and *triceps skinfold* (Houmard ***et***

al., 1991), is also important although the associations with serum lipoprotein-lipids level and hormones are less clear. It is postulated that thigh fat may contribute to lipoprotein profiles that predict lower risk of cardiovascular disease (Seidell *et al.*, 1989; Terry *et al.*, 1991).

II.1.2.3. Diet

Both total fat and fatty acid composition are important determinants of plasma cholesterol levels. Saturated fats raise the serum cholesterol and triglyceride levels whereas polyunsaturated fats cause them to decrease. In a small number of male subjects, changing the diet from high to low fat content for a short period of time (2 weeks), reduced the cholesterol level significantly (Reed *et al.*, 1987). However, recently it was found that stearic acid (C18, saturated fatty acid) is unique and has an independent effect of lowering plasma cholesterol level (*Derr et al.*, 1993). Genetic traits and the body's self-regulating metabolic mechanisms (e.g. rate-limiting enzymes in cholesterol synthesis and excretion) cause a marked variability of response between individuals (Quivers *et al.*, 1992). Vegetarians have lower cholesterol and non-HDL cholesterol than non-vegetarians (Sacks *et al.*, 1975; Masarei *et al.*, 1984), perhaps because the sterols of plant origin are not only very poorly absorbed but also inhibit the absorption of cholesterol, probably by competing with cholesterol for esterification with fatty acid, but possibly also due to the effects of different lengths and saturation of plant fatty acids and their effects on lipid metabolism.

II.1.2.4. Alcohol

A regular, moderate intake of alcohol will increase the level of HDL-C in plasma (Castelli *et al.*, 1977; Masarei *et al.*, 1986; Puddey *et al.*, 1986; Marinetti, 1990; Välimäki *et al.*, 1991). This effect is independent of all other variables. However, after acute ethanol administration, there is an increase in triglycerides without change in HDL-C (Mishra *et al.*, 1991).

II.1.2.5. Cigarette smoking

Cigarette smoking has a negative correlation with HDL-C, while a study on cigarette smoking cessation has shown an increase in plasma concentration of apolipoprotein A-II (Masarei ***et al.,*** 1991).

II.1.2.6. Exercise

Lipoprotein metabolism, especially the lipolytic rate of triglyceride-rich lipoproteins, is influenced by physical training, increasing with increasing activity. Vigorous activity in men will increase plasma HDL-C level as the result of an increase in the HDL_2 subfraction and apolipoprotein A-I. (Wood and Haskell, 1979; Kiens, 1992).

II.1.2.7. Gender differences

In Caucasians, females have higher total-HDL-C and HDL_2-C levels in plasma than males (Anderson ***et al.,*** 1978; Martini ***et al.,*** 1984; James ***et al.,*** 1989; James and Pometta, 1990; Atger ***et al.***, 1990). Females tend to have lower triglyceride, LDL-C, VLDL-C and higher HDL-C levels than men from puberty onwards until menopause but thereafter those in females become higher than in men of similar age, except those who take hormone replacement therapy (HRT) (Heiss ***et al.,*** 1980). This sex-differential variation in HDL_2-C may be due to lower levels of hepatic lipase (HL), resulting in a lower rate of removal of HDL-C, and faster triglyceride clearance due to higher lipoprotein lipase (LPL) in females. (Godsland ***et al.,*** 1987; Seed, 1991 for review). The sex-difference in HDL_2-C levels can be compensated for in part by exercise in men (Wood and Huskell, 1979). An *inverse* relationship between HDL_2-C and postprandial catabolism of triglyceride (Patsch ***et al.***, 1983), a *direct* relationship between HDL_2-C and lipoprotein lipase (Taskinen and Nikkilä, 1981, Patsch ***et al.,*** 1987), and an *inverse* correlation between HDL_2-C and hepatic lipase activity

(Patsch *et al.*, 1987) have been documented.

II.2. Sex Hormones

II.2.1. General concepts of sex hormone-production and the metabolism of sex hormones

Hormone concentrations in blood are kept relatively constant because the ratio between their secretion and disposal rates is rather constant. The secretion rate for a hormone is defined as the rate at which the hormone is secreted from the sum of the endocrine glands, which is expressed as the amount of hormone secreted per unit of time (e.g. μg/day). If the hormone is secreted by one gland, then the secretion rate is equal to production rate as is the case with cortisol and aldosterone. However, sex steroids such as estradiol and testosterone are secreted by the gonads and are also produced in peripheral tissues by the transformation of hormonally inactive steroid precursors through metabolic processes (extraglandular transformation). Therefore the production rate of a steroid hormone is equal to the sum of its secretion rate and transformation rate. On the other hand the metabolic clearance rate (MCR) is defined as the apparent volume of whole blood or plasma from which a substance is completely and irrevocably removed per unit of time. There is a close relationship between hormone binding in plasma, the hepatic extraction and MCR. If the MCR remains constant, a decrease in plasma concentration reflects accurately the decrease in production rate. However, if the binding capacity of serum is increased, then the MCR will be decreased, since tissue extraction and metabolism are reduced. The MCR of sex steroids is inversely related to the relative binding affinity to SHBG. (Lipsett, 1986).

SHBG, also known as TeBG (testosterone-estradiol binding globulin) and SBP (Sex steroid binding protein), is a circulating glycoprotein with molecular weight about 80,000 to 90,000. It is synthesized in the liver, and its biological function in the circulation is the transport of sex steroid hormones. It has high

affinity binding (10^9 L/mol) (Anderson, 1974), therefore, SHBG-bound steroids are not readily available for target tissue binding and action. However, it has a low capacity for sex-hormone binding, one binding site per mole (Yen, 1986).

Although the serum unbound or "free" fraction of sex steroids is readily available for biological action at the target cells, loosely albumin-bound fractions and the fraction represented by salivary sex steroids are also considered to be biologically active (Wong ***et al.***, 1990; Pardridge, **1991;** Swinkels ***et al.***, 1991). The binding affinity of SHBG for testosterone at 37°C is much greater than that of serum albumin. In comparison to the binding affinity for testosterone, the affinity of SHBG is about three times as great for DHT, and for estradiol only about one third. Androstenediol binds less strongly than does testosterone (Anderson, 1974). There is virtually no SHBG binding of androstenedione and DHEA (Yen, 1986).

II.2.1.1. Biosynthesis of testosterone

In men, the synthesis of testosterone is almost exclusively controlled by the hypothalamic-pituitary-testicular axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which stimulates the anterior segment of the pituitary gland, located at the base of the brain, to release luteinizing hormone (LH) into the blood stream. The LH then stimulates the Leydig cells of the testis to synthesize and secrete testosterone into the circulatory system. Cholesterol is the precursor of steroid hormones, and may be obtained from LDL-cholesterol in the blood or from *de novo* synthesis from acetate. In the human, the major biosynthetic pathway is via the $\Delta 5$-pathway including pregnenolone, 17-hydroxypregnenolone, and androstenediol. Testosterone and androstenedione are the end-products which are secreted into the blood and are converted to estradiol and estrone respectively, at extraglandular sites in peripheral tissues. Moreover, androstenedione can be converted to testosterone

(Lipsett, 1986). Thus the production rate of serum testosterone is the sum of secretion rate of testosterone and the production rate of testosterone from androstenedione.

II.2.1.2. Metabolism of testosterone

When testosterone enters a cell, it may be metabolized by two pathways. One is from testosterone → DHT → 5-α-androstenediol, of which the end-metabolites DHT and 5-α-androstenediol are more potent than testosterone at the tissue level. Another is from testosterone → androsterone → etiocholanolone, of which the end-metabolites are less potent and are conjugated in the liver and excreted in the bile or the urine. It is believed that both types of metabolism could occur in a sequential manner in the same cell. In either pathway, the metabolism of testosterone is accomplished by a change in steroid structure (Bardin, 1986). Non-conjugated, biologically active testosterone in circulation may also be filtered at the glomerulus and excreted in the urine (Lipsett, 1986).

In total, the outcomes of the metabolism of testosterone can be classified into three aspects (Bardin, 1986) as follows:

(1) The biopotency of testosterone is modified by aromatization to a potent estrogen, 17β-estradiol. This provides significant local concentrations of estrogens in individual tissues and contributes significantly to the blood estrogen level.

(2) The biological activity of testosterone is amplified by 5α-reductase which reduces the major blood androgen (testosterone) to the more potent DHT and 5α-androstenediol at the tissue level, especially in tissue of the male reproductive tract and skin.

(3) The biological activity of testosterone is modified by the action of 5β-reductase. The consequence of this reaction is to produce 5β-DHT and 5β-reduced steroid (etiocholanolone) which are then further metabolized to sulphate-

or glucuronide- conjugated 17-ketosteroids in the liver and excreted in the urine. Only about 1% of secreted testosterone is excreted in the urine as the testosterone-17β glucuronide (Horton, 1989).

Since substantial amounts of androstenedione and other adrenal androgens (such as DHEA and DHEA-sulfate) are also metabolized to 17-ketosteroids, it is now clear that the determination of total urinary ketosteroids is a poor reflection of testosterone secretion and androgenicity. However, the measurement of urinary total testosterone (the sum of unconjugated testosterone and testosterone-17β glucuronide and /or sulfate in urine) is an index of testosterone production in blood and in tissue (Camacho and Migeon, 1964; Korenman and Lipsett, 1964).

II.2.1.3. Dihydrotestosterone (DHT)

Although it is the most potent form of testosterone at the tissue level, it is only secreted in small amounts by the testis, and contributes very little to the overall androgen content of the blood in men. There is little free DHT in the circulation because it has stronger binding affinity to SHBG than does testosterone. Testosterone and androstenedione are precursors of plasma DHT. Testosterone accounts for at least 70% of plasma DHT in men. (Horton, 1989).

II.2.1.4. Androstenedione

Leydig cells secrete small amounts of androstenedione, and the remainder is secreted by the adrenal cortex or derived from dehydroepiandrosterone (DHEA) by peripheral tranformation (Bardin, 1986). The adrenal output of androstenedione is responsive to adrenocorticotropic hormone (ACTH) stimulation and exhibits a diurnal rhythm coincident with that of cortisol (Yen, 1986).

II.2.1.5. Biosynthesis of estrogen in men

Testes secrete estrogenic hormones, but under normal conditions account for only a negligible amount of that present in blood (Bardin, 1986).

The major portions of blood estradiol and estrone in normal men are derived from blood testosterone and androstenedione, respectively. This conversion of androgens to estrogens requires cytochrome p-450-dependent aromatase which is present in many non-endocrine tissues. (Bardin, 1986).

II.2.1.6. Metabolism of estrogen

The estrogens are preferentially conjugated with glucuronic acid, sulfates, and /or mixed conjugates, as well as other polar conjugates in the liver and excreted in the urine. (Lipsett, 1986).

II.2.2. Factors affecting sex hormone levels in plasma

Several variables should be borne in mind when one considers the levels of sex hormones in a subject.

II.2.2.1. Sex hormone binding globulin (SHBG)

Variation in sex hormone levels in adults may be due to in part the differences in SHBG levels. Adult women have twice the plasma concentration of SHBG of adult men. This sex difference is due to the fact that SHBG production is promoted by estrogen and inhibited by androgen. The level of circulating SHBG is considered a major controlling factor in the balance between biologically active androgens and estrogens (Anderson, 1974; Yen, 1986). SHBG levels in turn are affected by obesity, a variable which also shows strong associations with lipid levels, so obesity needs to be taken into account when evaluating associations between sex hormone levels and lipids.

This relationship of sex hormones and binding proteins is an important factor in the interpretation of circulating hormone levels and biologic action at target tissues.

II.2.2.2. Sampling time

The secretion of testosterone is episodic or pulsatile. (Yen, 1986; Smith

and Rodriguez-Rigau, 1989), and the same is true of estradiol, as most of the estradiol in men is derived from the aromatization of testosterone and androstenedione (Bardin, 1986). Single values of sex steroid concentrations should be interpreted with caution. Estimates may be improved by utilization of multiple or integrated blood-sampling techniques (Smith and Rodriguez-Rigau, 1989).

II.2.2.3. Stress and acute or chronic non-endocrine illnesses

In hemodialysed patients (Bogicevic and Stefanovic, 1988) and male patients with respiratory failure (Kouchiyama ***et al.,*** 1989), the serum level of testosterone is decreased. In kidney stone patients (Van Aswegan ***et al.,*** 1989), the urinary level of testosterone was lowered. The plasma DHEA-S and DHT concentration in young men is decreased after a myocardial infarction (Slowinska-Srzednicka ***et al.,*** 1989). In patients with septic shock, there is a dramatic increase in estrone and estradiol, associated with a decrease in testosterone in plasma (Christeff ***et al.,*** 1988). Persons with ischemic heart disease have significantly lower testosterone levels than persons without ischemic heart disease (Lichtenstein ***et al.,*** 1987). Patients with positive coronarographic findings have low levels of testosterone (Hromadová ***et al.,*** 1985). Serum total- and free- testosterone were 30% lower in hypertensive than in normotensive men (Hughes ***et al.,*** 1990). In patients with premature coronary vascular disease, testosterone concentration was significantly lower than that in normal healthy Indian men (Sewdarsen ***et al.,*** 1990). Overproduction of corticosteroids has been reported to suppress testosterone levels in normal adult males. The increased cortisol level due to surgical stress may contribute to the suppressed level of testosterone (Doerr and Pirke, 1976).

It is concluded that systemic illness has effects on plasma testosterone levels.

II.2.2.4. Ageing

It has been found that 24-hour mean integrated plasma testosterone levels are decreased in healthy older men (Zumoff ***et al.,*** 1982). Also in adult men the bioavailable testosterone levels decline significantly with age while those of total testosterone decrease less significantly (Dai, ***et al.,*** 1981; Nahoul and Roger, 1990). Some investigators have reported that the age-related reduction in free testosterone is due to the age-related increase in sex hormone-binding globulin capacity rather than a reduction of total serum testosterone. The consequent reductions in free testosterone are dispropotionate to those of total testosterone (Blackman, 1989). It is said that the urinary total testosterone is age dependent, as a distinct decrease in total urinary testosterone is observed in elderly persons (Van Aswegan ***et al.,*** 1989). The plasma level of DHEA-sulfate decreases with aging (Orentreich ***et al.,*** 1984).

II.2.2.5. Diet and nutrition

A western diet increases and a vegetarian diet decreases the urinary testosterone excretion in men and this is accompanied by changing serum lipoprotein profiles (Hill ***et al.,*** 1980).

It appears that energy surfeit has an effect on blood hormone concentrations as overfeeding female subjects results in weight gain with increased plasma somatomedin-C/insulin-like growth factor, testosterone and insulin (Forbes ***et al.,*** 1989). It has been demonstrated also that dietary lipid intake is an additional factor involved in the regulation of plasma levels of SHBG in normal men. After consuming a diet with a high fat content ($>$100g fat/day) for two weeks, the mean SHBG level decreases (mean total cholesterol level increases). Changing the diet from one with a high fat to low fat content ($<$20g/day) for a further two week period results in a significant reduction in

mean plasma cholesterol level, while the mean SHBG level increases. The increase in plasma SHBG is associated with a significant decrease in the free testosterone fraction and free testosterone concentration (Reed ***et al.,*** 1987).

II.2.2.6. Medication, drugs and alcohol

Antifungal drugs such as Ketoconazole and Fluconazole when taken orally will block adrenal steroidogenesis by inhibition of cytochrome p450-dependent enzyme (aromatase) (Contreras ***et al.,*** 1985; Devenport ***et al.,*** 1989). Feminization due to generalized increase of aromatase in peripheral tissues can be treated with an aromatase inhibitor (Bardin, 1986). Antiandrogens such as progesterone, cyproterone acetate, flutamide, or spironolactone generally demonstrate high receptor affinity but do not activate or lead to translocation of the antiandrogens-receptor complex. Most of these substances also suppress LH and thus reduce testosterone secretion (Horton, 1989).

It has been shown that athletes who take 110-116 grams of ethanol [about 2g/kg body weight] in six hours will increase the ratio between testosterone and epitestosterone in urine, most likely due to the increase of testosterone (Falk ***et al.,*** 1988).

II.2.2.7. Body composition and obesity

It was reported that morbidly obese men have higher estrogen and lower dehydroepiandrosterone sulphate (DHEAS), testosterone (total and free) and SHBG concentrations than control normal-weight subjects (Pasquali ***et al.,*** 1988). Zumoff ***et al.*** (1990) found that plasma calculated 'free' testosterone and non-SHBG-bound testosterone (both are biologically active) are decreased in obese men in proportion to their degree of obesity. However, early reports suggested that the reduction in total testosterone in moderately obese men is accompanied by a reduction in the level of SHBG, resulting in a normal level of free testosterone (Amatruda ***et al.,*** 1978). In non-obese females, increased androgenic

activity (ratio of free testosterone to total testosterone) and degree of obesity (BMI) are independently related to increased waist-hip ratio (Seidell ***et al.,*** 1989). It was also reported that plasma sex-hormone binding globulin and percent free testosterone are associated with regional adiposity in sedentary middle-aged men, but do not account for the correlations between WHR and lipoproteins (Terry ***et al.,*** 1989). Nevertheless, *it has been concluded that body composition as reflected by body mass index rather than body weight is associated with sex steroid alterations, with consequent changes in plasma lipoprotein profiles* (Segal ***et al.,*** 1987).

II.2.2.8. Variations in states of sleep-wake cycle

There is evidence for a circadian rhythm of testosterone secretion in young men. The acrophase of the rhythm is about the time of awakening, and the nadir is in the evening; the amplitude is small (10 to 25 per cent). There is a sleep-related rise of LH and testosterone level during puberty in boys. The circadian rhythm of testosterone in young adults may be a result of a persistent sleep-related rise in this steroid. It may ultimately disappear with advancing age (Bardin, 1986).

II.2.2.9. Levels of physical and sympathetic nervous system activity

Earlier studies showed that the urinary free and total testosterone levels in sportswomen were markedly higher in comparison with those of the inactive females. Recently several studies have indicated that exercise induces an acute elevation in the circulating testosterone levels, while exercise on a regular basis has been demonstrated to affect the resting levels of testosterone. Both the acute and long term testosterone responses to exercise are determined by the mode, intensity and duration of the exercise (Deschenes ***et al.,*** 1991; Galbo, 1991). It has been reported that endurance trained male athletes demonstrate a reduction in serum testosterone concentration (Hackney ***et al.,*** 1990). In contrast

to endurance training, weight-training has been demonstrated to increase resting levels of testosterone. It was found that significant correlations exist between increases in strength and elevations in the free to bound testosterone ratio (Deschenes, ***et al.,*** 1991 for review).

However, even a modest, achievable exercise program for men with premature myocardial infarction can also be beneficial by ameliorating their hyperestrogenemia and reducing atherogenic factors (Mendoza ***et al.,*** 1991).

Subjects with Type A behavior pattern exhibit a chronic excess discharge and circulation of catecholamines from nerve endings and hormones from the pituitary, adrenal and pancreatic glands. The overstimulating of the sympathetic nervous system leads to increased intravascular deposition of the clotting elements of the blood and an excess of the insulin in their blood indicating abnormal disposition of fat and sugar metabolism in the body (Friedman & Rosenman, 1975). Although elevated day time urinary excretion of testosterone glucuronide in men with the type A behavior pattern was found (Zumoff ***et al.,*** 1984), it was reported that type A and type B behavior patterns are not correlated with testosterone concentration in serum (Dai ***et al.,*** 1981).

Interestingly, factors affecting both sex hormone levels and lipoprotein-lipids are similar and may imply a relationship between sex hormones and lipoprotein-lipids.

II.3. The relationship between sex hormones and lipoproteins

II.3.1. Gender difference in sex hormones, menopause and lipoprotein-lipids

The association between hyperlipidaemia and atherogenesis is well established biochemically both from human and animal studies. Much epidemiologic data indicates that the concentrations of plasma lipoprotein-lipids

may be better predictors of risk for atherosclerosis than are the levels of total plasma lipids alone (Miller, ***et al.,*** 1981, 1987; Johansson ***et al.,*** 1991; Drexel ***et al.,*** 1992, Levinson and Wagner, 1992). Levels of HDL-C among white Americans are similar in boys and girls before puberty. As boys mature sexually, their levels of HDL-C , especially the HDL_2 fraction, fall below those of girls and remain so throughout the whole of adult life. In contrast, there is no such drastic change in girls, their levels of HDL_2 remaining high until the menopause. After the menopause, the levels of HDL-C and HDL_2-C fall, and the levels of total cholesterol, total triglyceride, and LDL-C increase except in those who take hormone replacement therapy (HRT) (Heiss ***et al.,*** 1980). This unique lipoprotein profile in menopause/postmenopausal women is demonstrated by epidemiologic studies (Kuller ***et al.,*** 1990; Jensen ***et al.,*** 1990; Stevenson ***et al.,*** 1993) and is believed to be mediated by estradiol. Concomitantly, the incidence of CAD in males is higher than in pre-menopausal females, and the incidence of CAD in postmenopausal women is higher than that of premenopausal. Raised serum LDL-C levels are also noted in surgical menopause (bilateral oophorectomy) (Farish ***et al.,*** 1990). It is reported that postmenopausal women who are on HRT have less risk of mortality due to CHD (Knopp, 1988; Barrett-Connor and Bush, 1991). The protective effect of estrogen may be mediated through increased HDL-C levels (Bush ***et al.,*** 1987; Mattews ***et al.,*** 1989), although other beneficial effects on arterial wall by preventing development of aortic and coronary atheroma are also proposed (Adams ***et al.,*** 1990).

II.3.2. Interventional study, exogenous sex hormone and lipoprotein-lipids in men

In an interventional study in a small number of normal men, the suppression of plasma testosterone by a long-acting gonadotropin releasing hormone analog ($LHRH_A$) leads to an increase in serum total cholesterol, HDL-C,

and apolipoprotein AI and B (Goldberg ***et al.,*** 1985). Unlike the effect of androgenic steroids as used by healthy young athletes, which results in a fall of serum HDL-C, intramuscular administration of exogenous, aromatizable testosterone (testosterone enanthate) does *not* induce androgen-induced hepatic triglyceride lipase activity *nor* decrease the HDL-C level (Friedl ***et al.,*** 1990). However, in a study of a group of young sterile testosterone-deficient men, treatment with exogenous methyl testosterone orally restored the level of testosterone, and improved the initial dyslipidaemia due to testosterone deficiency. The lipid profiles were normalized with the restored testosterone level with decreasing triglyceride, LDL-C, apoB and increasing HDL (Hromadová ***et al.,*** 1991).

II.3.3. The relationships of endogenous sex hormones and lipoprotein-lipids

Masarei ***et al.*** (1980) documented that endogenous plasma estradiol has a weak *positive* correlation with HDL-C, while plasma SHBG is strongly *positively* correlated with HDL-C in both post- and pre-menopausal women. The subsequent studies performed by the same group (Semmens ***et al.,*** 1983) found SHBG is independently, directly associated with HDL-C in men and women and suggested that there is an *inverse* correlation between unbound endogenous testosterone and HDL-C. Plasma estradiol levels are not significantly associated with HDL-C in either sex. Stefanick and co-workers (1987) showed that plasma total testosterone levels have significantly *inverse* associations with HDL cholesterol and HDL_2 mass concentrations, and a significant *negative* relationship was found between SHBG and triglycerides, while the plasma E_2 level was found independently, *inversely* related to total and LDL-cholesterol. There is *no* clear relationship between E_2/T and HDL-C after adjusting for other confounders.

However, the data of other workers pursuing this subject showed a

different result and suggested a *direct* relationship between free testosterone and HDL-C (Heller ***et al.***, 1983). Lichtenstein ***et al.*** (1987) proposed that the serum E_2 levels are associated *directly* with HDL, but the relationship disappeared after adjusting for the testosterone and insulin level, whereas the serum testosterone levels are associated directly with HDL and inversely with triglyceride. The associations persist after adjusting for age, BMI, insulin and/or triglyceride.

Kiel ***et al.*** (1989) presented a *negative* result showing that there is *no* association between total- or free- testosterone and HDL-C, whereas a consistently *positive* correlation exists between total estradiol or calculated free estradiol and both total cholesterol and HDL-C. In addition, they proposed that there is a complex interaction between endogenous testosterone and estradiol with respect to total cholesterol. Khaw and Barrett-Connor (1991) carried out a community cohort study from which they concluded that HDL-C levels are *positively*, and VLDL-C levels *negatively* associated with serum testosterone, after adjusting for confounders. Serum E_2 levels are *positively* correlated with LDL-C. However, in the Multiple Risk Factor Intervention Trial (MRFIT) Dai ***et al.*** (1981) demonstrated an *inverse* relation between plasma estradiol levels and LDL-C concentration in spite of a consistent direct relation between plasma testosterone and HDL-C concentrations in both cross-sectional and longitudinal analyses among men.

Since unbound rather than SHBG-bound sex hormones are bioavailable (Anderson, 1974), SHBG in circulation functions as a modulator of sex-hormone delivery to tissue (Nestler, 1993). Adiposity has effects on SHBG concentrations and sex-hormone levels (Bray, 1989). Furthermore, obesity is an important source of variation in lipoprotein-lipids levels and profiles. There may be associations between endogenous sex hormones and lipoprotein-lipids when these potential confounders are accounted for.

Chapter III. Materials and Methods

III.1. Subjects and Sampling Methods

A total of 46 apparently healthy Chinese men, aged 19 to 53 years, were recruited for the study of the association between sex hormones and lipoproteins. Due to the intraindividual variability of lipoprotein-lipids and the diurnal oscillations of sex hormones, sampling was carried out on two occasions to try to account for some of this variation. 24 hr urine samples were collected twice (22 ± 10 days apart), and fasting blood samples were drawn the following morning between 8:30 and 10:00 am. after fasting from 9 pm the night before, 19 ± 8 days apart. Lipoprotein-lipids, apolipoproteins, sex hormones, and sex hormone-binding globulin in serum samples were determined. Serum testosterone and estradiol concentrations and urinary unconjugated and conjugated testosterone and estradiol together with unconjugated (free) cortisol were also determined. Each determination was performed in duplicate.

No subject was taking drugs or medications or was engaged in any unusual physical activity prior to blood taking. The degree of adiposity expressed as body mass index (BMI) was determined as weight/height^2 (kg/m^2). Body fat distribution was assessed as the waist to hip ratio (WHR), by measuring minimal waist and maximal hip girth. Alcohol consumption, and cigarette consumption were recorded.

Blood was drawn by venipuncture of the antecubital vein in the seated position, with a 21-gauge needle attached to a 20 ml plastic syringe and collected without any anticoagulant into two plastic tubes. After standing at room temperature for 1 to 2 hours, the serum was separated by centrifugation (1,500× g, 5 min, 4°C). The serum was removed and aliquots were prepared in small, screw-capped, plastic vials. All vials were snap-frozen in liquid nitrogen immediately and then stored at -70°C. Venus blood collected without

anticoagulant was processed without undue delay to remove RBC, since red cells at room temperature will alter plasma concentration of active steroid hormones; they can degrade estradiol to estrone, and cortisol to cortisone as well as adsorb testosterone (Tietz[1], 1986).

An aliquot of the serum sample was used for gamma glutamyl transferase (γGT) determination as a marker for excessive alcohol consumption, and for serum albumin for the purpose of calculating unbound free hormones.

Urine was collected in plastic urine bottles without any preservatives added. After collection was finished urine samples were dispensed into glass tubes (13×100mm) in aliquots of 6 to 8 ml, capped tightly and stored at -70°C without any delay. Urinary creatinine was assayed in parallel with urinary hormones (Gerlo ***et al.***, 1991) to ensure the completeness of the 24-hr urinary collection. Urinary collections with <0.5 g of creatinine were considered incomplete 24-hr collections and were excluded. No specimen had a creatinine of <0.5g and therefore all were included.

The following biochemical analyses was generally performed in duplicate to reduce the effect of analytical variation.

III.2. Quantitation of serum lipoprotein-lipids

III.2.1 Determination of cholesterol and triglyceride

Total plasma cholesterol and triglyceride were determined enzymatically on a Cobas Bio analyzer (Roche Diagnostics, MontClair, New Jersey) using 2 point-standards (8.68 mmol/L and 4.34 mmol/L). The lower standard was derived by diluting stock (8.68 mmol/L) with 4% bovine albumin in aqueous solution.

The principle of cholesterol analysis involves the enzymatic hydrolysis of cholesterol ester to free cholesterol and fatty acids by cholesterol esterase and enzymatic oxidative conversion of free cholesterol to cholesterol-4-ene-3-one and

hydrogen peroxide by cholesterol oxidase. The hydrogen peroxide formed is then quantified by reaction with phenol and 4-aminophenazone in the presence of peroxidase to form an o-quinoneimine, which was measured photometrically at the wave length 520nm.

The principle of triglyceride analysis is based on four separate reactions: (1) enzymatic hydrolysis of triglycerides to glycerol and free fatty acid by lipase; (2) enzymatic conversion of glycerol and ATP to glycerol-3-phosphate and ADP by glycerol kinase; (3) oxidative conversion of glycerol-3-phosphate to di-hydroxy acetone phosphate and hydrogen peroxide by glycerol phosphate oxidase, and (4) conversion of the chromogen by hydrogen peroxide to quinoneimine using peroxidase, which can be measured photometrically at the wave length of 520nm.

The reagents were from Roche Diagnostic System (Basel, Switzland). Unimate 5 (lot S2832) and Unimate 5 (lot S1031) were used for cholesterol and triglyceride respectively, and Calibrator (Lot 1542) was used for both cholesterol and triglyceride. Ciba Corning ch-B , Lot N020103 and lot L025103, were used for quality control throughout all analyses.

A listing of parameter settings is attached as **Appendix i.** The intra- and inter-batch variations of controls at two different levels for cholesterol and triglyceride are summarized in **Table III.2.1.A** and **Table III.2.1.B.**

Table III.2.1.A. Intra-assay variations for cholesterol and triglyceride by enzymatic determination on Cobas Bio automatic analyzer using Unimate 5 reagent (Roche Dignostic System)

Parameter	Controls (mmol/L) (Mean ± SD)	No. of within-assay	Observed value (Mean ± SD)	Bias %	CV%
Cholesterol	3.6±0.5*	10	3.2±0.04	11.1	1.26
	2.7±0.4#	10	2.64±0.03	2.2	1.31
Triglyceride	0.9±0.1**	10	0.96±0.01	6.6	1.1
	1.8±0.3##	10	1.80±0.03	0	1.9

*,** QCSNORASY (Ciba Corning, lot no. 020103)
#,## QCSABNASY (Ciba Corning, lot no. 025103)

Table III.2.1.B. Inter -assay variations for cholesterol and triglyceride by enzymatic determination on Cobas Bio automatic analyzer using Unimate 5 reagent system (RocheDiagnostic System)

Parameter	Controls (mmol/L) (Mean ±SD)	no. of between assay	Observed value (Mean ± SD)	Bias %	CV%
Cholesterol	3.6±0.5*	8	3.35±0.15	6.9	4.3
	2.7±0.4#	8	2.58±0.16	4.4	6
Triglyceride	0.9±0.1**	8	0.94±0.03	4	3.5
	1.8±0.3##	8	1.95±0.08	8	3.9

*,** QCSNORASY (Ciba Corning, lot no. 020103)
#,## QCSABNASY (Ciba Corning, lot no. 025103)

III.2.2. Determination of HDL-Cholesterol and its subfractions

Lipoprotein concentrations in serum are usually measured in terms of their cholesterol content. HDL-Cholesterol and its subfractions were determined by measuring cholesterol in the supernatant subsequent to dual precipitation with the precipitating agent--$MgCl_2$/Dextran sulfate (Warnick ***et al.,*** 1982; Bachorik and Albers, 1986). The first precipitation was to remove apoB-containing lipoproteins by mixing serum samples (500 µl) well with Reagent A (50 µl) and letting it stand at room temperature for 15 min. The precipitate was then removed by centrifugation at 2,000x g for 15 min, and the cholesterol in the supernatant determined. The subsequent precipitation was achieved by adding Reagent B (25 µl) to the HDL-C supernatant (250 µl) at room temperature and letting it stand for 20 min. HDL_3-C was then determined in the supernatant. Appropriate dilution factors were applied for each step in order to get the final concentrations. The reagent A is a mixture of 1% dextran sulfate (50,000 MW) and $MgCl_2$ (0.5 mol/L). The reagent B is a mixture of 1% dextran sulfate and $MgCl_2$ (1.5 mol/L).

HDL_2-cholesterol concentrations were calculated by subtracting HDL_3-cholesterol from the total HDL-cholesterol.

The reagents used for cholesterol determination were similar to those for total serum cholesterol, except for a lower level of 3-point standards (0.54, 1.08, 2.17 mmol/L respectively), which were obtained by serial dilution from a stock

calibrator (8.64 mmol/L) with 4% bovine albumin in aqueous solution. Also, sample volumes were increased from 5 μl to 20 μl for better precision and recovery. QCsNorasy (Ciba Corning lot no.N020103) was used for quality control. After precipitation with $MgCl_2$/Dextran sulfate, the coefficient of intra-assay coefficient of variation for using this QC material was 1.6%, and for interassay was 8.4% **(Table III.2.2)**.

Table III.2.2. Intra- and inter-assay variation for HDL-cholesterol

QC for HDL assay (QCsNorASY)★ → ↓	**Within-assay (n=12)**	**Between-assay (n=12)**
Expected value (Mean±SD) (mmol/L)	0.9±0.2	0.9±0.2
Observed value (Mean±SD)(mmol/L)	0.89±0.014	0.88±0.07
Bias %	1	2
CV%	1.6	8.4

★ Ciba Corning (lot no.020103)

III.2.3. Determination of VLDL-C and LDL-C

These values were derived by applying the formula of Friedewald ***et al.*** (1972). This formula is based on two assumptions: (i) that most of the fasting triglyceride in plasma is located in VLDL (ii) that the mass ratio of triglyceride:cholesterol in VLDL is 5:1 (molar ratio 2.19:1). This is true except in type III hyperlipoproteinaemia or in the presence of marked hypertriglyceridaemia (triglyceride>5.0mmol/L). Thus, the value (in mmol/L) of VLDL-C is calculated by dividing triglyceride by 2.2 and LDL-C is obtained from the equation :

LDL-C = Total cholesterol - HDL-cholesterol - Triglyceride/2.2 (All in mmol/L).

III.2.4. Quantitative determination of serum apolipoproteins and Lp(a)

III.2.4.1. Determination of Apolipoproteins A-I and B

ApoA-I and apoB levels were measured by immunonephelometry using the Beckman Array nephelometer (Beckman Instruments, Brea, Calif.). The reagents used were from Beckman: ApoA (lot no. M010221) and ApoB (lot no. M011173). The assay is calibrated against Beckman calibrator (Beckman lot M105114), and controlled with a control serum from Beckman (lot M109227).

The rate nephelometer measures the peak rate of changes in intensity of light as it is scattered by particles which are formed by the immunoprecipitin reaction of a specific antibody and the specific antigen in suspension. The coefficients of variation within run and between runs were $< 5\%$ and $< 8\%$, respectively.

III.2.4.2. Determination of Lipoprotein (a)

Lp(a) was measured by the SPQ test system for Lp(a) (Incstar Co., Stillwater, Minn. USA) on a Cobas Bio automatic analyzer. The principle is based on immunoturbidimetric analysis. The antigen-antibody reaction will produce turbidity in the mixture and thus increase the amount of light absorbed by the solution. After an incubation period lasting approximately 10 min, the absorbance of the solution is measured at a wavelength of 340 nm. A calibration curve is generated by assaying a series of standards with known concentrations of lipoprotein (a) and by using the data reduction capability of the Cobas Bio (Dens plot). Concentrations for the control and samples are interpolated from the calibration curve. The lowest detection limit of the standard curve is 55 mg/L. The coefficients of variation within run and between runs were $< 3\%$ and $< 9\%$, respectively.

III.3. Quantitative determination of sex hormones

As there are no suitable commercial immunoassays available for the determination of very low levels of unconjugated testosterone and unconjugated

estradiol in the urine, sensitive in-house radioimmunoassays for the two hormones were set up.

III.3.1. For urinary unconjugated and serum total testosterone

Anti-testosterone serum was raised in the rabbit to the testosterone-3-(o-carboxy methyl) oxime-BSA conjugate. It was a gift to Dr. N. S. Panesar from the Division of Steroid Encocrinology, Leeds University, England and had been kept in our laboratory since 1985.

[1,2,6,7-^{3}H]-Testosterone (Amersham code TRK.402, batch 58, 1985) was kindly provided by Dr. N. S. Panesar. It needed to be purified before use (See III.3.1.1). After this batch of the tracer ran out a new batch of [1,2,6,7-^{3}H]-Testosterone (Amersham code TRK.402, batch 85, 1992) was purchased from Amersham Co. England, which was used without further purification. All tracers were diluted with assay buffer to give an activity of 10,000 dpm/0.1 ml .

A series of non-radioactive testosterone (Sigma T-1500) solutions was made up in absolute ethanol so that each 100μl contained 0, 43.4,.86.8, 173.6, 347.2, 694.4 fmol of testosterone respectively. These solutions served as standards in RIA and the ethanol was evaporated before assay.

Phosphate buffer (0.05 mol/L) containing 0.1% gelatin and 0.01% sodium azide with pH adjusted to 7.4 was used for diluting anti-testosterone serum and tracer throughout. Dextran-coated charcoal (0.5%) which contains activated charcoal (Sigma, no.C-5385) and dextran (BDH chemical Ltd., Poole, England) was used for removing the excess free testosterone during assay. The charcoal (2.5g) and dextran (0.25g) were mixed in 500 ml phosphate buffer on a magnetic stirrer for one hour. After mixing, the preparation was stored at 4°C.

III.3.1.1 Experimental Procedures

Determination of the optimal antibody titre

An antiserum dilution curve **(Fig. 1)** was obtained by incubation of a fixed amount of tracer ligand (0.1 ml) with 0.1 ml of various dilutions of the antiserum with or without unlabelled testosterone added. Usually the highest concentration of the standard was used. The optimal titre could be determined by the largest displacement of these two curves near 50%-bound of radioactivity. In practice 1:8000 and 1:16,000 dilutions were used.

Establishment of a standard curve and quality controls

Preparation of standards

Starting with 1mg/ml of non-radioactive testosterone in absolute ethanol as a stock, serial dilutions were made with absolute ethanol to give a final concentration of 2.0 ng/ml which was the highest standard (694.4 fmol/100µl). From 2.0 ng/ml, doubling dilutions with absolute ethanol were made to get solutions of 347.2, 173.6, 86.8, and 43.4 fmol per 100μl. Absolute ethanol was used as a zero standard. All standards were kept separately in screw-capped glass vials and stored at -20°C to minimize evaporation. The standards were brought back to room temperature before use.

The typical standard curve for testosterone was obtained by incubating a known concentration of antiserum, standards, and a fixed amount of ^{3}H-testosterone (10,000 dpm) at 4°C for 16 hours The free (unbound) testosterone was adsorbed by addition of 0.5 ml cold dextran-coated charcoal and centrifugation at 3,500 g for 15 min at 4°C. The supernatant was decanted into a vial containing 5 ml scintillation cocktail and radioactivity determined in a β-spectrometer. The %bound radioactivity was calculated, and plotted as %bound of radioactivity vs testosterone concentration **(Fig.2)**.

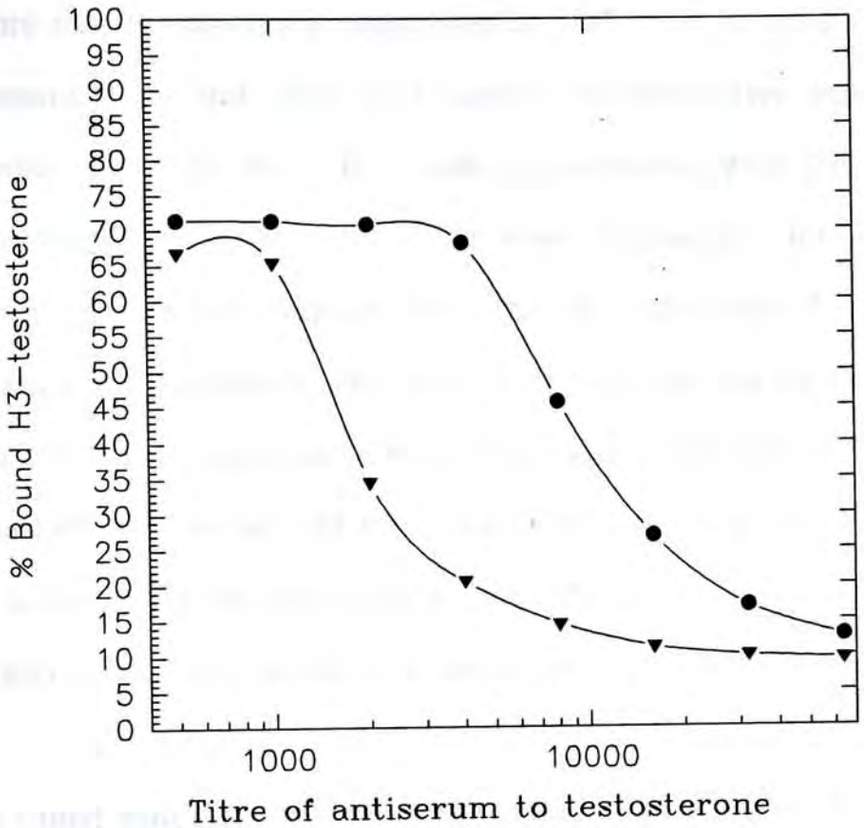

Fig. 1. Testosterone antiserum dilution curve

A fixed amount of tritiated testosterone (^{3}H-testosterone) (10,000 dpm/0.1ml) was incubated at 4°C with a fixed amount (0.1ml) of various dilutions of rabbit anti-testosterone serum with or without cold testosterone (200 pg) added. After an incubation period of 16 hours, the excess free triatiated testosterone was absorbed by dextran-coated charcoal.

▼ - ▼ curve obtained with cold testosterone added. • - • curve obtained without any cold testosterone. The optimal titre of antiserum could be determined by the largest displacement from these two curves near 50% bound.

Purification of radioactively-labelled ^{3}H-testosterone

The purity of the tracer could be restored after long storage by thin-layer chromatography. Half a sheet of aluminium TLC sheet precoated with silica gel 60 F254 (size: 20×20cm, layer thickness 0.22 mm) (Merck Art.5554) was used. Before use, the sheet was reactivated at 110°C for 30 mins. 10μl of 1 μci/μl of ^{3}H-testosterone and 10μl of 1 mg/ml non-radioactive testosterone were run together vertically in a TLC tank pre-saturated with the solvent system of cyclohexane:ethylacetate (60:40) at room temperature for 135 min until the solvent reached one centimeter from the top of the sheet. The chromatogram was air-dried in a fumehood. The dried chromatogram was then examined under UV light. Non-radioactive testosterone fluoresces under light of 265nm and served as a marker. The average Rf for testosterone after three determinations was 0.38. (Rf is defined as the ratio of the distance the sample moved from the origin to the distance the solvent moved from the origin in the chromatogram.).

Once the position of ^{3}H-testosterone was determined, it could be cut out and eluted with a known amount of absolute ethanol. The eluent was centrifuged to remove the silica gel and 0.1 ml was transferred into a scintillation counting vial containing 2ml of scintillation fluid, and the radioactivity counted. The eluent was evaporated under a nitrogen stream and redispersed in a known amount of phosphate buffer. This was used as a stock solution. Before use, it was diluted with phosphate buffer to give the desired radioactivity.

Preparation of charcoal- stripped urine as zero calibrator (blank)

Activated charcoal and an aliquot of urine (or plasma) (10g per 100ml) were stirred at 4°C in a cold room overnight (Chard, 1990). For charcoal-stripped urine, the mixture was filtered through Whatman No.1 filter paper twice, and then through a Millipore 0.22μ filter twice to remove all the charcoal particles. For charcoal- stripped plasma, it was necessary to centrifuge the mixture twice at high speed (10,000×g) for one hour before passing through the

Millipore filter, as the charcoal particles carried by the plasma passed through Whatman No.1 filter paper. The final clear filtrate was then prepared in aliquots and stored at -70°C.

Preparation of spiked urine or plasma

The testosterone stock solution (1mg/ml) was diluted 1:1000 to obtain a final concentration of 1 μg/ml. 100 μl of this solution was added to 100 ml stripped urine (or plasma) to make 347.2 fmol/100 μl spiked urine (or plasma). The subsequent 173.6 fmol/100μl and 86.8 fmol/100 μl spiked urine or plasma solutions were obtained by doubling dilution of the 347.2 fmol/100 μl solution with charcoal-stripped zero calibrator. All spiked urine or plasma samples were dispensed in aliquots of 0.3ml (assay volume) in borosilicate glass tubes (100×13mm), capped tightly and stored at -70°C.

Preparation of samples for RIA

For urine samples, usually 1.5 ml of the clear supernatant was used after the crude urine was centrifuged at a speed of 1,500g for five minutes. Diethylether (3ml) was added to the urine sample in a 100×13mm borosilicate glass tube. The extraction was carried out by vortexing vigorously on a multitube vortexer at speed 4 for 60 seconds, three times. The organic and aqueous phases were then separated by freezing the aqueous phase at -70°C for 20 mins. One ml of the upper layer (ether extract) was transferred to a 10×75 mm glass tube (in duplicate) for RIA. The urine volume was reduced if the concentration was too high.

Serum samples were diluted 1:10 with 0.9% saline before extraction. 0.3 ml of the diluted serum was extracted with 3 ml of diethylether. One ml of the ether extract was transferred to a glass tube (in duplicate) for RIA.

RIA

All standards, samples, and controls were processed identically. Two charcoal-stripped blank tubes were also included, not only for monitoring the binding of antiserum at zero concentration but also for monitoring the non-specific binding in buffer. The protocol was as follows:

1). 10×75mm glass tubes were labelled for non-specific binding, blank, controls and samples in duplicate, and standards in triplicate.

2). 1 ml of the ether extract or 0.1 ml of standards were transferred to each corresponding glass tube.

3). 0.1 ml of absolute ethanol was added to all tubes of samples and controls, and 1 ml of diethylether was added to all tubes containing standards.

4). The tubes were evaporated in vacuum oven at 40°C under a pressure of 30 mmHg for 45 to 60 minutes.

5). 0.1 ml of antiserum was added to all tubes except tubes for non-specific binding which had 0.1 ml buffer added instead and the tube vortex mixed at speed 3 for 20 seconds on a multitube vortexer.

6). 0.1 ml of tracer was added to all tubes and the tubes vortex mixed at speed 3 for 20 seconds.

7). Incubation was performed at 4°C for 16 hours.

8). 500 μl of cold dextran-coated charcoal was added to and tubes vortex-mixed gently at speed 2 for 20 seconds. This step was performed on ice. All tubes were standing on ice for 15 mins to reach equilibrium after the dextran-coated charcoal was added to the first tube.

9). The tubes were centrifuged for 15 mins. at 4°C, ×3,500g.

10). The supernatant was decanted into a scintillation counting vial containing 5 ml scintillation fluid. The contents were mixed vigorously and counted in the scintillation.

Calculation

The standard curve was plotted as % bound of total ^{3}H-testosterone radioactivity vs concentration of the standard. The concentration of testosterone in the unknown sample was interpolated from the standard curve, and the results were corrected for the dilution factor.

III.3.1.2. Characteristics of the radioimmunoassay for testosterone

Sensitivity

The sensitivity (limit of detection) of an assay is usually defined as twice the standard deviation of the blank determination on a standard curve (Haning ***et al.,*** 1979; Chard, 1990).

Ten replicates of zero standard (expressed as %bound of total count) were used to calculate the mean of counts bound and the standard deviation (i.e. 39.8 +/- 3.3%). A new standard curve was also constructed by plotting the mean of counts bound of ten replicates of all standards as the function of testosterone concentration. As shown in **Fig.2**, the mean plus/minus two S.D. (33-46% bound) is considered as the confidence limits of the zero standard while the mean of the zero standard minus two S.D. (40-33% bound), read as concentration (i.e.12 pg or 41.6 fmol per 0.1 ml ethanol), is the minimum detection limit of this standard curve. Also the mean and the standard deviation of the corresponding 10 replicates of charcoal-stripped urine blank (which is 41 +/- 3.5%) were calculated. The corresponding "limit of detection" is about 10 pg or 34.7 fmol per 0.1ml of biological fluid which is slightly lower than that of the zero standard of pure ethanol. This slight difference is probably due to a matrix effect, which could be overcome by increasing the levels of sex hormones in the assayed biological fluids to well above the minimal detection limit. In this study the levels of sex hormones in assayed biological fluids were many times greater

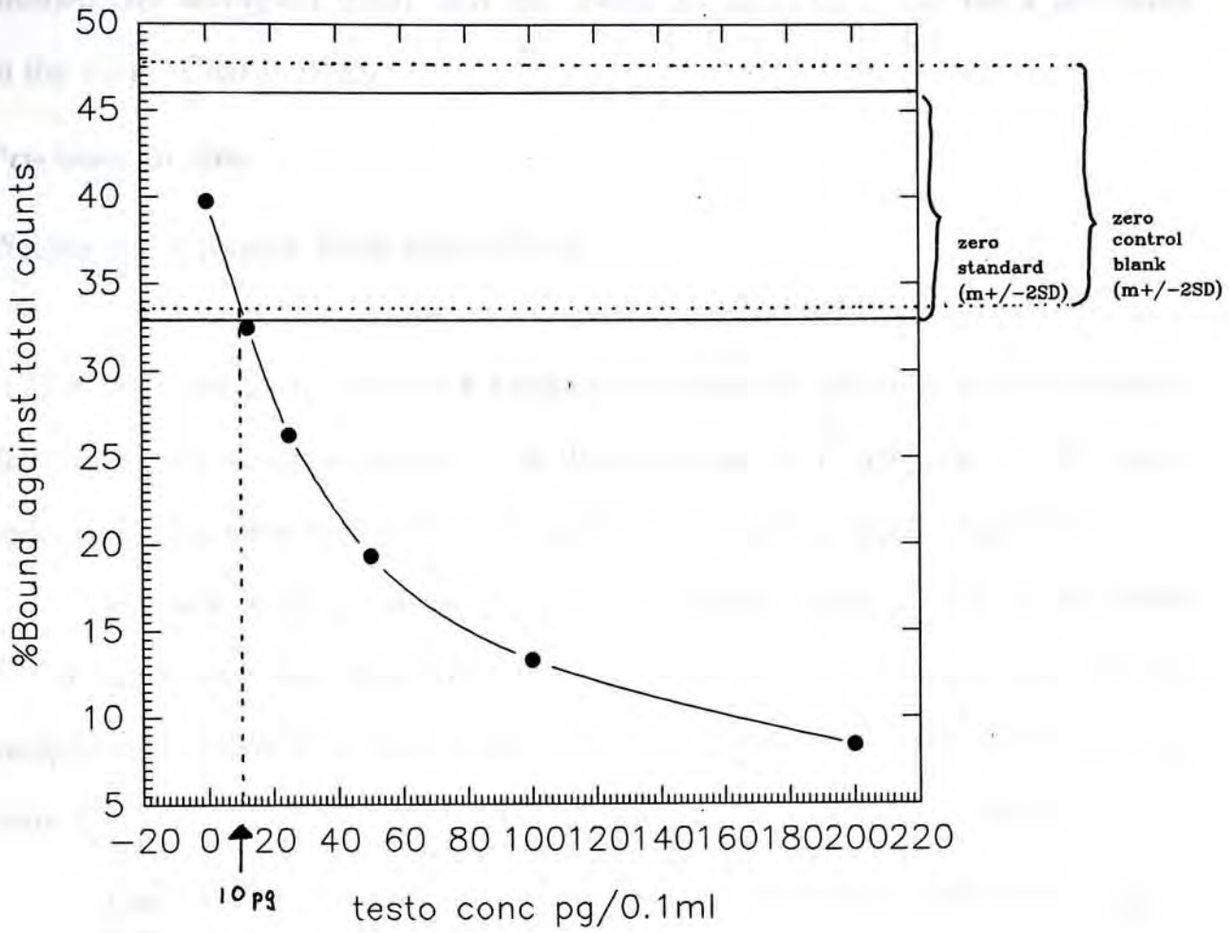

Fig. 2. The lowest detection limit of the standard curve for testosterone

A new standard curve of ten RIA replicates was constructed by plotting the mean counts (as % bound) of all standards in these ten replicates as the function of testosterone concentration (pg/0.1ml). The confidence limit (mean+2SD) to the zero standard estimate was 46.4%, while the lowest detection limit of this standard curve (mean-2SD) read as concentration was about 12 pg/0.1ml (at 33.2% bound). Similarly the lowest detection limit using this standard curve by the zero control blank (charcoal-stripped urine) was also calculated, which was about 10 pg/0.1ml (at 33.6% bound), whereas the confidence limit to the zero blank estimate (mean+2SD) was 47.6%. The lowest detection limit of the zero blank in biological fluid is 2 pg lower than that of pure ethanol. In this case, the slight difference of lowest detection limit in these two different systems could be overcome by increasing the levels of sex hormone in assayed biological fluids well above the minimal detection limit.

than the minimal detection limit [usually between 50-100 pg (173.6-347.2 fmol)/0.1ml biological fluid], and the lowest detection limit was not a limitation in the assay (Chard, 1990).

Precision studies

Within- and between- batch imprecisions

The spiked charcoal-stripped urine with high (347.2 fmol/tube), medium (173.6 fmol/tube), or low (86.8 fmol/tube) values of testosterone were used to determine within- and between- batch imprecisions. 14-17 sets and 30-31 sets of spiked samples were used for within- and between-batch assays respectively.

Between batches, the spiked charcoal-stripped urines at 347.2 fmol/tube, 173.6 fmol/tube, and 86.8 fmol/tube respectively were spaced out between samples and always processed in the same way as that of samples. The variations were noted and summarized in **Table III.3.1.2.A** and **Table III.3.1.2.B.**

Table III.3.1.2.A. Within-run variation for assay of testosterone added to charcoal-stripped urine.

Testosterone added fmol (pg)	Volume of charcoal-stripped urine blank	N	CV %
347.2 (100)	0.1ml	17	5.7
173.6 (50)	0.1ml	14	7.6
86.8 (25)	0.1ml	17	7.3

Table III.3.1.2.B. Between-run variation of assays of testosterone added to charcoal-stripped urine

Testosterone added fmol (pg)	Volume of charcoal-stripped urine blank	N	CV %
347.2 (100)	0.1ml	31	7.25
173.6 (50)	0.1ml	31	7.38
86.8 (25)	0.1ml	30	7.98

Recoveries

The recoveries of spiked control urines at different concentrations were calculated by the formula: %Recovery = Mean of the testosterone measured ÷Total testosterone spiked ×100%. The results were summarized in **Table III.3.1.2.C**.

Table III.3.1.2.C. The recoveries of known amounts of testosterone added to charcoal-stripped urine, between immunoassays.

Testosterone spiked fmol (pg)	Testosterone measured (Mean ±SEM) pg	N	Recovery Rate %
347.2 (100)	101.94 ± 1.42	17	101.94
173.6 (50)	53.85 ± 1.09	14	108
86.8 (25)	27 ± 0.48	17	108

Test of linearity

For this purpose, a urine pool which showed a low level of testosterone was used. Urine samples were prepared in aliquots of 0.3, 0.6, 0.9 and 1.2 ml in glass tubes which were respectively extracted with a fixed amount (3ml) of diethylether. 0.1 ml of each ether extract was used for RIA. The amount of steroid recovered showed a linear relationship with urine volume (**Fig. 3**).

Comparison with another procedure

The in-house testosterone assay was compared with the commercial assay (DPC) using the sera from the 22 healthy men. The correlation between these two methods was good ($r=0.933$, $p=0.000$); $RIA_{inhouse}=1.19\times RIA_{dpc}+0.02$ (**Fig.4**). The value obtained by the "in-house" assay is higher than that of the DPC commercial kit, which may be due to the different specificities of the two antisera, or to small differences in calibration.

In view of the agreement between these two methods, the decision to assay serum testosterone with the in-house assay method was therefore made for economic reasons and the consistency of the analytical methods between urine

and serum.

Cross reactivity of the antiserum

Specificity is a characteristic of immunoassay. However, some biological materials may interfere in the radioimmunoassay by competing with the ligand at

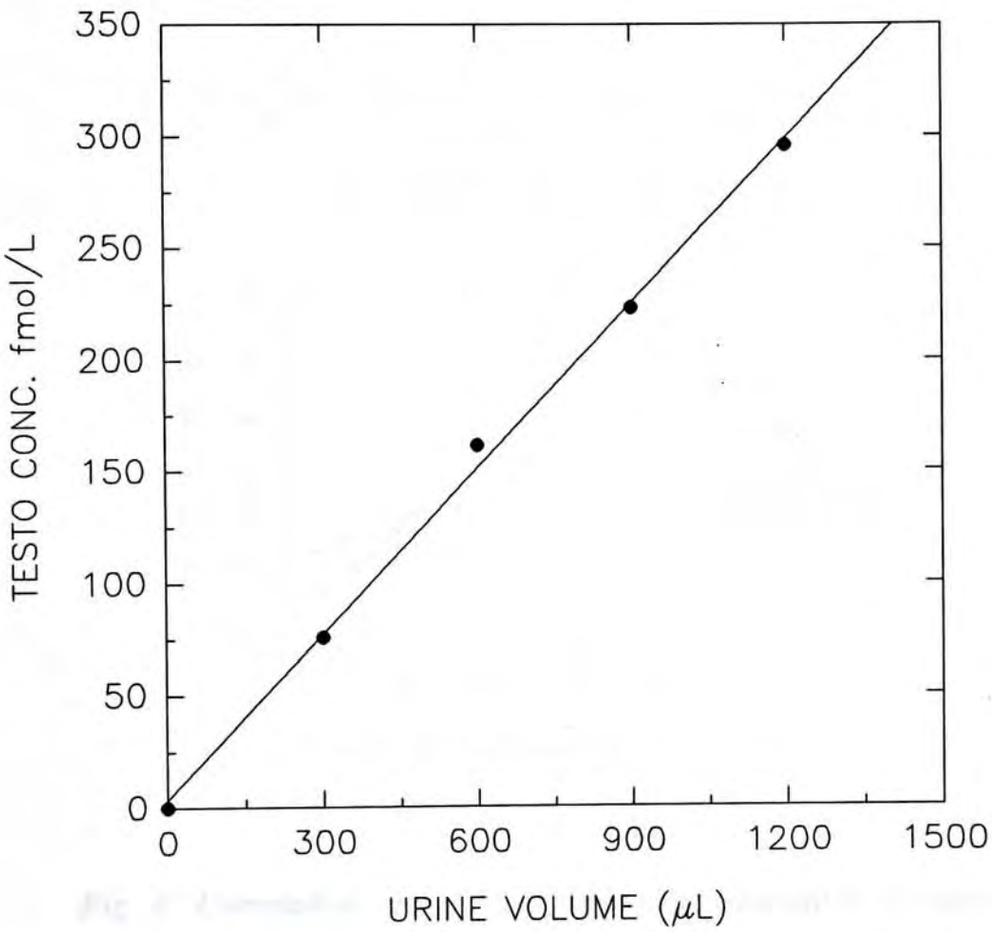

Fig.3. The dose-response relationship between testosterone concentration and urine volume when assayed by the "in house" RIA.

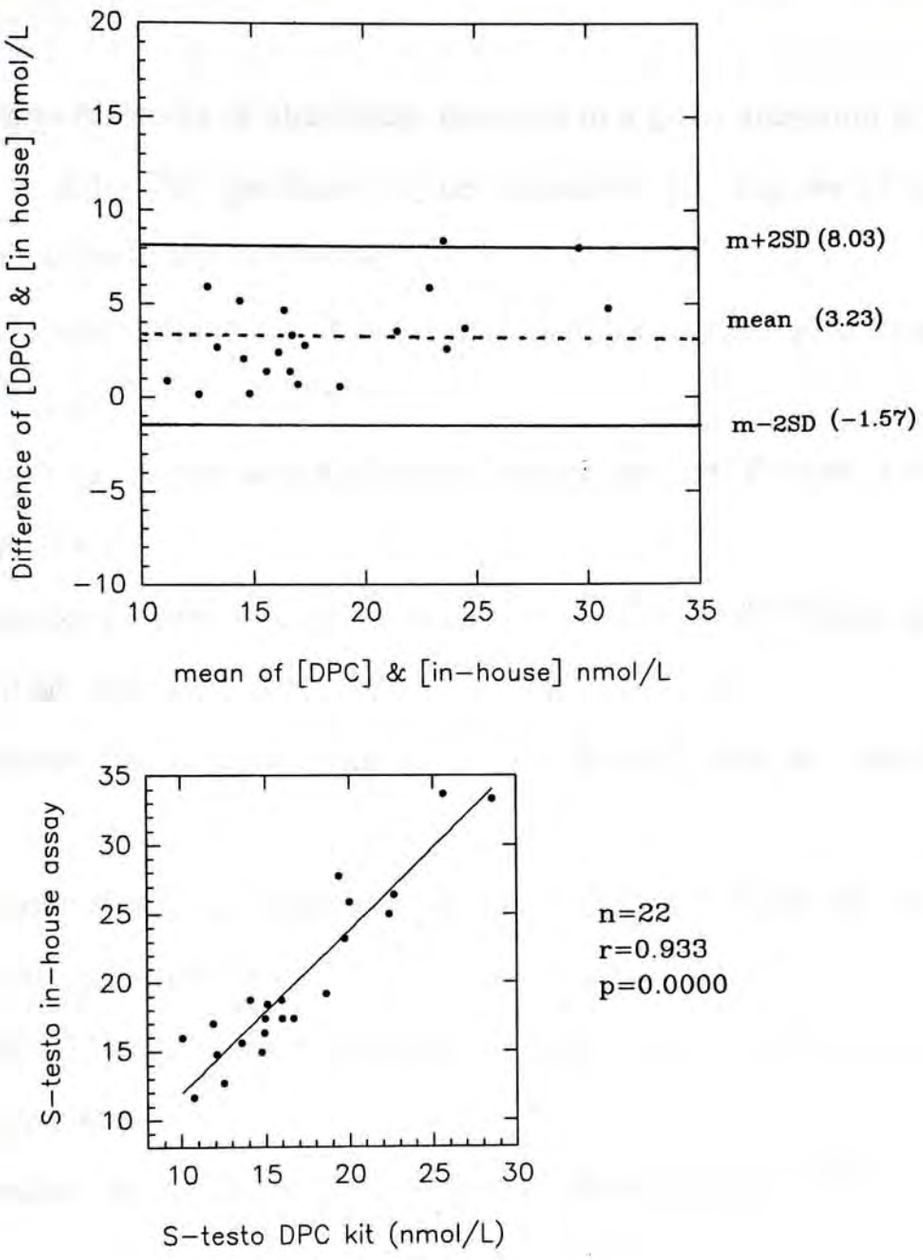

Fig. 4. Correlation of results of serum testosterone in 22 apparently healthy Chinese men, which were determined by "in-house" RIA (y axis) and DPC RIA kit (x axis). Y=1.19 X + 0.02 (r=0.933, p=0.0000). The value obtained by " in-house" RIA is higher than that by DPC kit. (bottom). Another way to express the correlation (top) was by plotting the difference in values between the two methods against their mean, with discrepancies of up to 8 nmol/L. Mean=3.23 nmol/L and SD=2.4 nmol/L. A subject with a extremely high value was excluded. The discrepancies might be due to different standardization of the method and the specificity of the antiserum prepared.

the binding site of the antibody due to their physicochemical resemblance in structure .

Cross-reactivity of identifiable materials to a given antiserum is one of the methods to judge the specificity of the antiserum. The sources of structurally similar compounds are as follows:

1. d-aldosterone (4-pregnen-18-al-11ß,21-diol-3,20-dione), Sigma catalogue no. A-6628, Lot no. 23F-4025, FW 360.5
2. Progesterone (4-pregnene-3,20-dione), Sigma cat. no. P-0130, Lot no. 73F-0198, FW 314.5
3. 17-hydroxy-progesterone (Δ4-pregnene-17-ol-3,20-dione), Sigma cat. no. H-5752, Lot no. 83F-0458, FW 330.4
4. Hydrocortisone (cortisol), Sigma cat. no. H-4001, Lot no. 14F-0064, FW 362.5
5. Corticosterone (Δ^4-pregnene-11ß, 21-diol-3,20-dione) Sigma cat. no. A- 9630, Lot no. 18C-0226, FW 284.4
6. 5-DHT (Δ^5-androstan-17ß-ol-3-one), Sigma cat. no. A-8380, Lot no. 14F-0469, FW 290.4
7. ß-Estradiol, Sigma cat. no. E-8875, Lot no. 34F-0540, MW 272.4.

Procedure

a. Serial dilutions of a stock solution (1 mg/ml) in absolute ethanol from all compounds in question were made as follows: 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, ...up to 0.095 ng/ml.

b. 0.1 ml of the diluted solution was put through RIA along with testosterone standards.

c. %bound vs concentration of testosterone was plotted on semi-log graph paper.

Percent cross-reaction at 50% displacement of testosterone standard curve was calculated from the formula:

$$\% \text{ Cross reactivity} = \frac{\text{Concentration of Testosterone (50\% bound)}}{\text{Concentration of Cross- reactant (50\% bound)}} \times 100$$

The cross-reactivity of the testosterone antiserum with other adrenal steroids such as d-aldosterone, 17-OH-progesterone, hydrocortisone (cortisol), corticosterone, and 17ß-estradiol was not traceable (with no reaction up to each mass of 10 ng/0.1ml) but there was 40% cross reaction with 5α-DHT, 0.27% with androstenedione and 0.015% with progesterone. The data are summarized in **Table III.3.1.2.D.**

Table III.3.1.2.D. Cross reactivity of some naturally occurring steroids with testosterone antiserum

Compound	Molecular weight	% Cross Rx at 50% displacement	Molar % cross reaction
Testosterone	288.4	100	100
5α-DHT	290.4	40	40.2
Androstenedion	286.4	0.27	0.27
Progesterone	314.5	0.014	0.015
d-aldosterone	360.5	-	-
17-OH-progesterone	330.4	-	-
Hydrocortisone	362.5	-	-
Corticosterone	346.5	-	-
17β-estradiol	272.4	-	-

Most (at least 70%) of plasma DHT in males is from testosterone and DHT is a form of potent metabolite of testosterone at the tissue level (Lipsett, 1986). The production rate of DHT in males is less than 5% of that of testosterone (Bardin, 1986). DHT has much higher (about three times) binding affinity to SHBG than that of testosterone (Lipsett, 1986). In view of these arguments one can assume that there would be very little cross-reaction from DHT in the assay of urinary and plasma testosterone.

III.3.2 For urinary total testosterone

Most of the metabolites of testosterone are in the form of glucuronide and/or sulfate conjugates which are more polar and soluble in water and therefore

excreted in the urine by glomerular filtration. For determination of urinary total testosterone, therefore, it is necessary to hydrolyze the conjugates with enzyme or acid before extraction and quantitation. The simplest method is by acid hydrolysis. However, acid hydrolysis may introduce the so called "non-specific non-specificity" (Chard, 1990) effect to the antiserum, resulting in interference and non-reproducibility. A traditional way to eliminate non-specific non-specificity is to dilute the sample. From several trials both with the "in house" and DPC RIAs and from the example of the protocols for urinary free cortisol and urinary total testosterone by DPC reagent kits, it was found that diluting the hydrolyzates 1:25 with charcoal-stripped urine prior to extraction could overcome this problem. The protocol developed for urinary total testosterone assay was as follows:

1. Acid hydrolysis: To 1 ml urine sample, 0.2 ml of 12N HCL was added at 100°C in a boiling water bath for 15 mins.
2. The resulting acid hydrolyzate was diluted 1:25 with charcoal-stripped urine.
3. Usually 0.3-0.6 ml of the diluted hydrolyzate (reduced volume if the concentration was too high) was extracted with 3ml diethylether. 1ml of the ether extract was used for RIA following the same procedure as that described in section **III.3.1.1.**

Test of linearity and recovery

To 1 ml of spiked urine (containing testosterone 5 ng/ml), 0.2 ml of 12N HCL was added and hydrolyzed in a boiling water bath for 15 mins. A charcoal-stripped urine blank was treated the same way. The resulting acid hydrolyzate was diluted 1:25 with charcoal-stripped urine. 0.3 ml, 0.6 ml, 0.9 ml, and 1.2 ml of the diluted hydrolyzate and the blank were extracted respectively with 3 ml of diethylether. 1 ml of the ether extract was used for RIA. As shown in **Fig.5** there was a linear relationship between measured steroid concentration and the

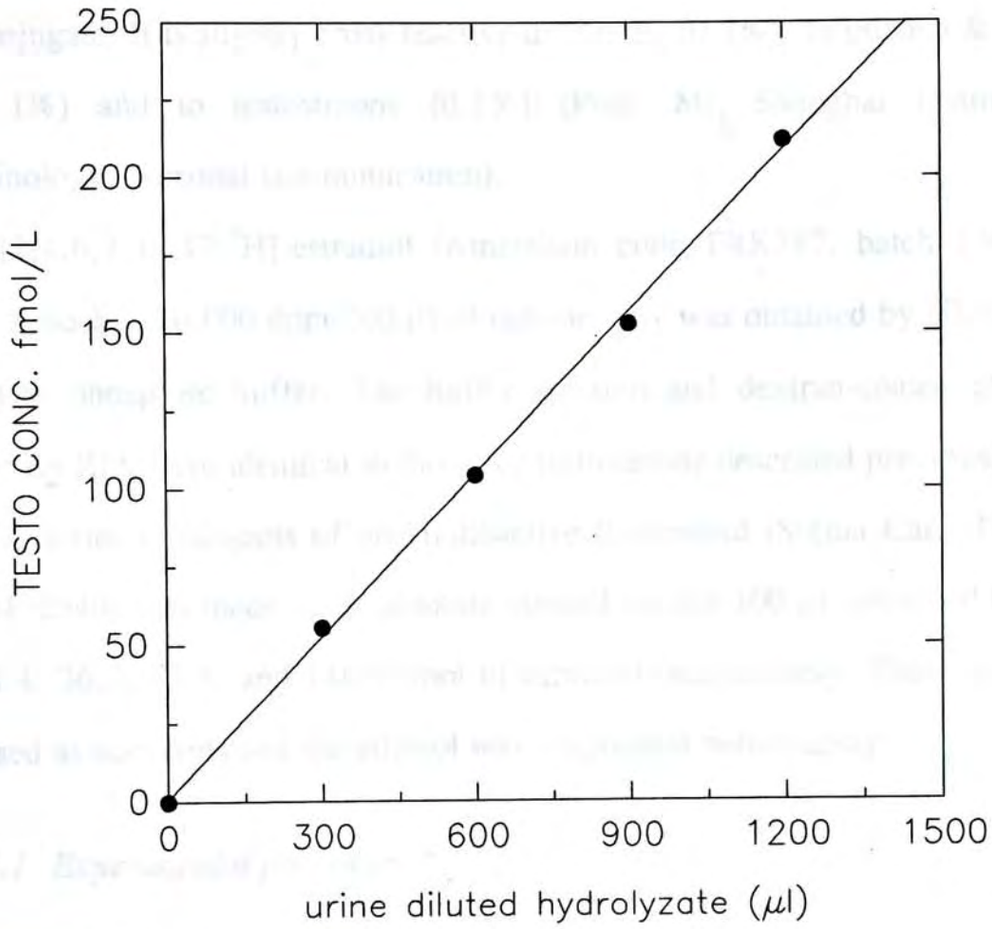

Fig. 5. The relationship between measured testosterone concentration and the volume of urine acid hydrolyzate diluted with charcoal-stripped urine.

volume of diluted urine hydrolyzate with a recovery of 91.3%.

III.3.3. For urinary unconjugated and serum total 17β-Estradiol

Lyophilized antiserum for 17β-E_2 was purchased from Shanghai Institute of Endocrinology, Shanghai, China. According to the information provided, this antiserum was raised in a rabbit against a 17β-estradiol-6-(carboxymethyl)-oxime-BSA conjugate. It is slightly cross-reactive to 17α-E_2 (0.7%), to estrone & estriol (about 1%) and to testosterone (0.1%) (Prof. Mi, Shanghai Institute of Endocrinology, personal communication).

[2,4,6,7,16,17-^{3}H]-estradiol (Amersham code TRK587, batch 114) was used as a stock. 20,000 dpm/100 µl of radioactivity was obtained by diluting the stock with phosphate buffer. The buffer solution and dextran-coated charcoal used for E_2 RIA were identical to those for testosterone described previously.

A series of aliquots of non-radioactive β-estradiol (Sigma Cat. E-8875, Lot 34F-0540) was made up in absolute ethanol so that 100 µl contained 0, 4.6, 9.2, 18.4, 36.7, 73.4, and 146.9 fmol of estradiol, respectively. These solutions were used as standards and the ethanol was evaporated before assay.

III.3.3.1 Experimental procedure

Determination of the optimal antibody titre

Antiserum dilution curves (**Fig.6**) were constructed by incubation of a fixed amount of tracer ligand (20,000 dpm/0.1ml) with 100 µl of various dilutions of the antiserum with and without standard (36.72 fmol/0.1ml) added. The optimal concentration was determined by the largest displacement from these

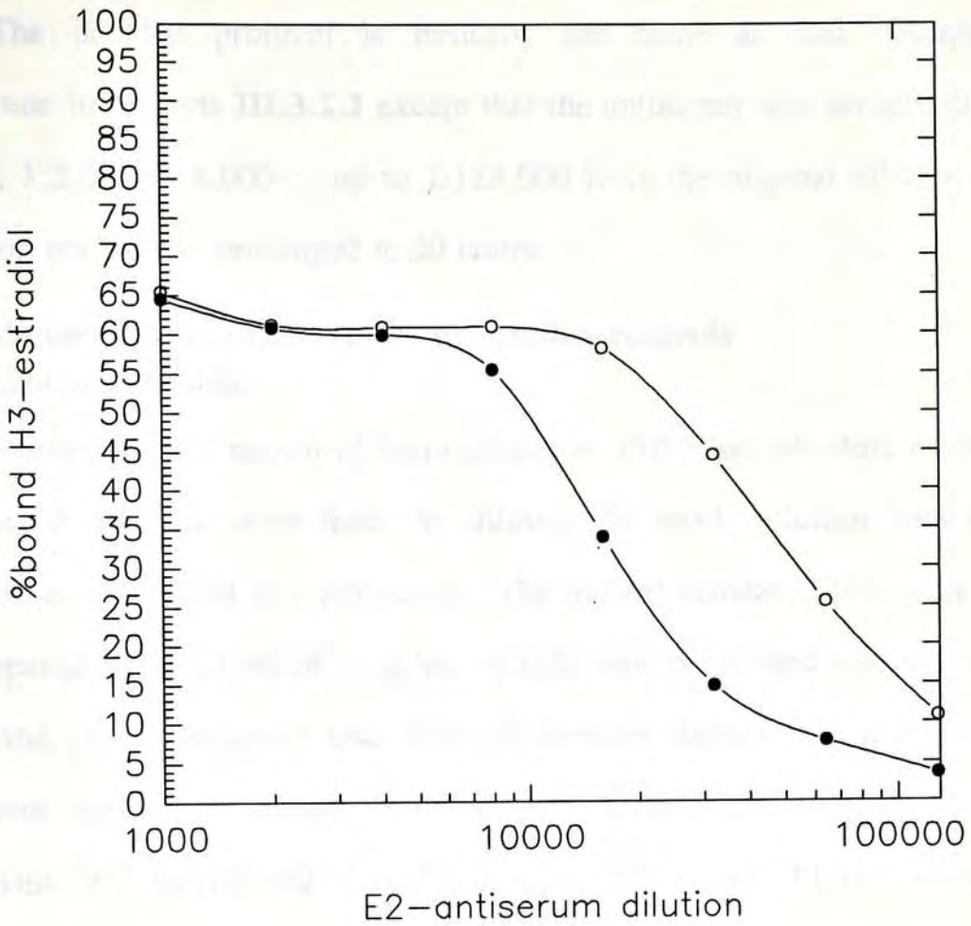

Fig. 6. The dilution curves of antiserum to 17β-estradiol.

∘ – ∘ without addition of cold E_2 ; • – • with cold E_2 (10 pg) added. A fixed amount of ^{3}H-17β-E_2 (20,000 dpm) was incubated at 4°C with a fixed amount of various dilutions of rabbit anti-17β-E_2 serum with or without cold 17β-E_2 (10 pg). After an incubation period of 20 hrs. the excess free ^{3}H- 17β-E_2 was absorbed by dextran-coated charcoal. The added cold E_2 competed with ^{3}H-E_2 at the binding site of antiserum, therefore, with less % bound of radioactivity the curve (• – •) was shifted to left. The optimal titre of antiserum could be determined by the largest displacement from these two curves.

two curves. In practice, a 1:40,000 dilution was used thoughout the experiments.

The detailed protocol is basically the same as that described for testosterone in sections **III.3.1.1** except that the antiserum was serially diluted to 1:1,000, 1:2,000, 1:4,000 ....up to 1:128,000 from the original dilution and the incubation period was prolonged to 20 hours.

Establishment of a standard curve and quality controls

Preparation of standards

Starting with 1 mg/ml of non-radioactive 17β-E2 in absolute ethanol as a stock, serial dilutions were made by diluting the stock solution with absolute ethanol to make 1ng/ml as a sub-stock. The highest standard, 146.9 fmol/0.1ml was prepared from 10 ml of 1 ng/ml, which was evaporated under a nitrogen stream and then redispersed into 25ml of absolute ethanol in a glass vial. The subsequent series of standards (73.4 fmol/0.1ml, 36.7 fmol/0.1ml, 18.4 fmol/0.1ml, 9.2 fmol/0.1ml, and 4.6 fmol/0.1ml) were achieved by doubling dilution from 146.9 fmol/0.1ml. Absolute ethanol was used as zero standard.

All standards were stored in glass vials with caps tightly screwed and kept in –20°C. All standards were brought back to room temperature before use.

Preparation of tracer ^{3}H-estradiol and construction of a standard curve

^{3}H-E_2 was diluted directly from the reagent bottle with phosphate buffer. About 20,000 dpm per tube were used throughout all assays.

A typical standard curve for E_2 RIA is shown as **Fig.7**. The detailed protocol was similar to that for testosterone, except the incubation period was prolonged to 20 hours which allowed the very minute amount of E_2, antiserum, and tracer to reach equilibrium completely at 4°C.

Preparation of spiked control urine or plasma

The base for spiked standards which served as controls was charcoal-stripped urine or plasma as described in Section **III. 3.1.1.**

E_2 sub-stock solution (1 ng/ml in absolute ethanol) was used for this purpose. The procedure is briefly described below:

4 ml of (1 ng/ml) substock solution was placed in a glass vial, dried under a nitrogen stream and then redispensed in 20 ml of charcoal- stripped urine or plasma. This gave a 73.4 fmol/0.1ml control solution which was double diluted to obtain 36.7 and 9.2 fmol standards. All spiked urines or plasmas were dispensed into 100×13mm borosilicate glass tubes as aliquots of 0.3 ml (assay volume), and stored at -70°C.

Preparation of samples for RIA

For urine samples, 1.5 ml of clear supernatant was used after the crude urine was centrifuged at 1,500g for 5 min to remove precipitates. For serum samples, 200 μl was used. Each sample was placed in a 100×13mm borosilicate glass tube and then extracted with 3 ml diethylether. 1 ml of the ether extract was used for RIA as described previously.

III.3.3.2. Characteristics of the radioimmunoassay for E_2

Sensitivity

As in the case of testosterone, twelve replicates of zero standard (expressed as % bound of total counts) were pooled and the mean of counts bound and the standard deviation (i.e. 41 +/- 1.5%) calculated. A standard curve was also constructed by plotting the mean of %bound of 12 replicates of all standards as the function of E_2 concentration. By the same token, the mean of the zero standard plus two S.D. (i.e. 41-44%) was considered as the confidence limit of the zero standard, while the mean of the zero standard minus two S.D. (i.e. 41-38%) read as concentration (i.e. 2 pg or 7.3 fmol of E_2 per 0.1 ml of ethanol) was the minimum detection limit of this standard curve. Moreover, the mean and standard deviation of the 12 replicates of charcoal-stripped urine blank

was about 41.2 +/- 1.7% which was quite close to the lowest detection limit of this standard curve (**Fig.7**). Again, the levels of sex hormone (E_2) in assayed biological fluids were orders of magnitude above the minimal detection limits [usually in the range of 10 to 20 pg (36.7 to 73.4 fmol)/0.1ml of biological fluids], so the lowest detection limit in this situation was not crucial (Chard, 1990).

Precision studies

Within- and between- batch imprecisions

The within-batch variations of control urines at high (73.4 fmol/tube), medium (36.7 fmol/tube), and low (9.2 fmol/tube) levels were noted. Between batches, the spiked controls at high, medium, and low levels were always processed in the same way as for urine samples. The coefficients of variations are summarized in **Table III.3.3.2.A** and **Table III.3.3.2.B**.

Table III.3.3.2.A. Within-run variation of known amount of E_2 added to charcoal-stripped urine.

17β-estradiol spiked fmol (pg)	Volume of charcoal-stripped urine blank	N	CV%
73.4 (20)	0.1ml	20	5.3
36.7 (10)	0.1ml	20	4.5
9.2 (2.5)	0.1ml	20	21

Table III.3.3.2.B. Between-run variation of known amount of E_2 added to charcoal-stripped urine.

17β-estradiol spiked fmol (pg)	Volume of charcoal-stripped urine blank	N	CV%
73.4 (20)	0.1ml	17	10.5
36.7 (10)	0.1ml	17	9.7
9.2 (2.5)	0.1ml	17	25

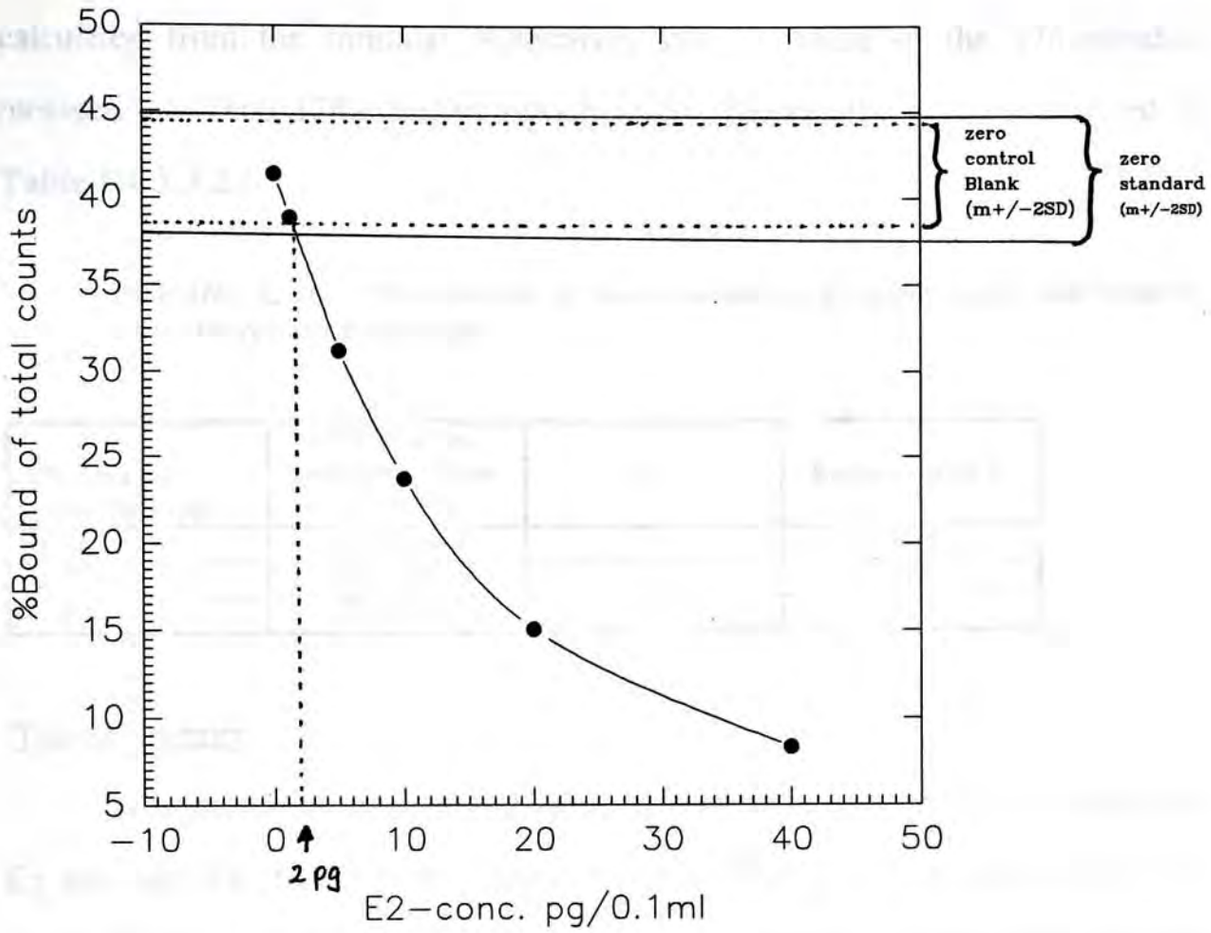

Fig. 7. The lowest detection limit of the standard curve for estradiol

A new standard curve of twelve RIA replicates was constructed by plotting the mean counts (as % bound) of all standards in these twelve replicates as the function of testosterone concentration (pg/0.1ml). The confidence limit (mean+2SD) to the zero standard estimate was 44.6%, while the lowest detection limit of this standard curve (mean-2SD) read as concentration was about 2 pg/0.1ml (at 37.8% bound). Similarly the lowest detection limit using this standard curve by the zero control blank (charcoal-stripped urine) was also calculated, which was about the same (38.4% bound), whereas the confidence limit to the zero blank estimate (mean+2SD) was 44.4%. The levels of estradiol in assayed biological fluids were manipulated in orders of magnitude above the minimal detection limit, therefore, the lowest detection limit in this case was not crucial.

Recoveries

The recoveries of spiked control urines at different concentrations were calculated from the formula: %Recovery rate = Mean of the 17ß-estradiol measured ÷ Total 17ß-estradiol spiked ×100. The results were summarized in **Table III.3.3.2.C**.

Table III.3.3.2.C. The recoveries of known amount of E_2 added to charcoal-stripped urine, between immunoassays

17ß-estradiol spiked fmol (pg)	17ß-estradiol measured (Mean ±SEM) pg	N	Recovery Rate %
73.44 (20)	18.07 ± 0.2	20	90.35
36.72 (10)	9.56 ± 0.09	20	95.6
9.18 (2.5)	2.19 ± 0.1	20	87.6

Test of linearity

A urine pool which showed a high concentrations of urinary unconjugated E_2 was used for this purpose. Urinary unconjugated E_2 was extracted with 3 ml of diethylether at various volumes of urine, and the relationship between measured E_2 and urine volume is shown in **Fig.8**.

Comparison with another procedure

The method was compared with an established commercially-available method, Amerlite (Amersham).

Experimental procedure:

1. A spiked plasma containing 1101 pmol/L (1101 fmol of E_2/ml) was prepared, and 5 aliquots were then assayed for E_2 by the Amerlite procedure which is based on enhanced luminescence competitive immunoassay and used for routine purposes at Prince of Wales Hospital.
2. In parallel, serial dilutions from 300 pg/ml (1101 fmol/ml) with the charcoal-stripped plasma blank were made to get concentrations of 150 pg/ml (551

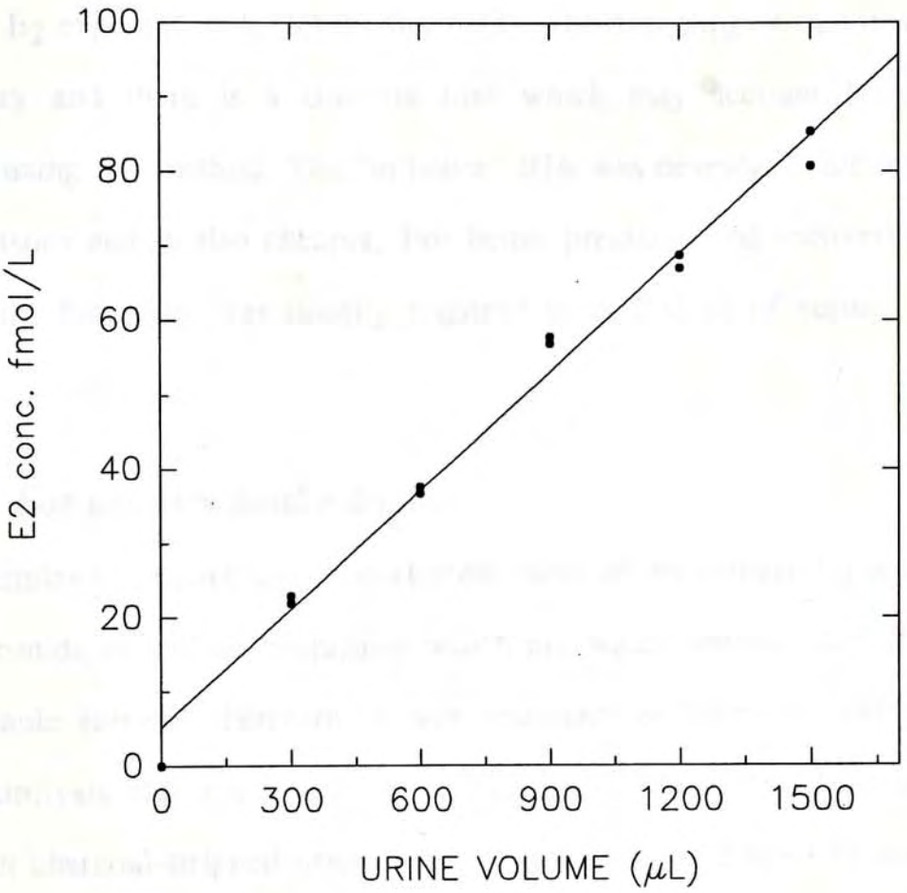

Fig. 8. The recovery of unconjugated E_2 using various volumes of urine after extraction with diethylether.

fmol/ml), 100 pg/ml (367 fmol/ml), and 50 pg/ml (184 fmol/ml). 5 samples of each dilution were assayed by the "in house" E2 RIA.

Recovery in the Amerlite procedure was 98.2%, and that for the "in house" assay 106% for both 100 pg/ml (367 fmol/ml) and 150 pg/ml (551 fmol/ml), and 116% for 50 pg/ml (184 fmol/ml). The regression line for recovery and expected value is shown in **Fig.9** with the equation: E_2 observed = 1.0085 × E_2 expected + 6.783 (r=0.99972). The data suggest that this method is satisfactory and there is a constant bias which may account for the higher recovery using this method. The "in house" RIA was developed due to its desired characteristics and is also cheaper. For better precision and recovery, increased sample size for assay was usually required (e.g. 200 μl of serum, 1.5 ml of urine).

III.3.4. For urinary total estradiol

Similar to urinary total testosterone, most of the urinary E_2 is in the form of glucuronide or sulfate conjugates which are water soluble. Before extraction with organic solvent, therefore, it was necessary to hydrolyze the conjugates. Acid hydrolysis was employed. After hydrolysis, the hydrolyzate was diluted 1:25 with charcoal-stripped urine blank. 0.6 ml of the diluted hydrolyzate was then extracted with 3 ml diethylether. One ml of the ether extract was then used for RIA as described previously.

Test of linearity and recovery

To 1 ml of spiked urine (3 ng/ml), 0.2 ml of 12N HCL was added and hydrolysis was carried out in a 100°C water bath for 15 mins. The resulting hydrolyzate was diluted with the charcoal-stripped urine blank at the ratio of 1:25. The final diluted hydrolyzate was then prepared in aliquots of 300μl, 600μl, 900μl per tube. All tubes were extracted with 3 ml of diethylether,

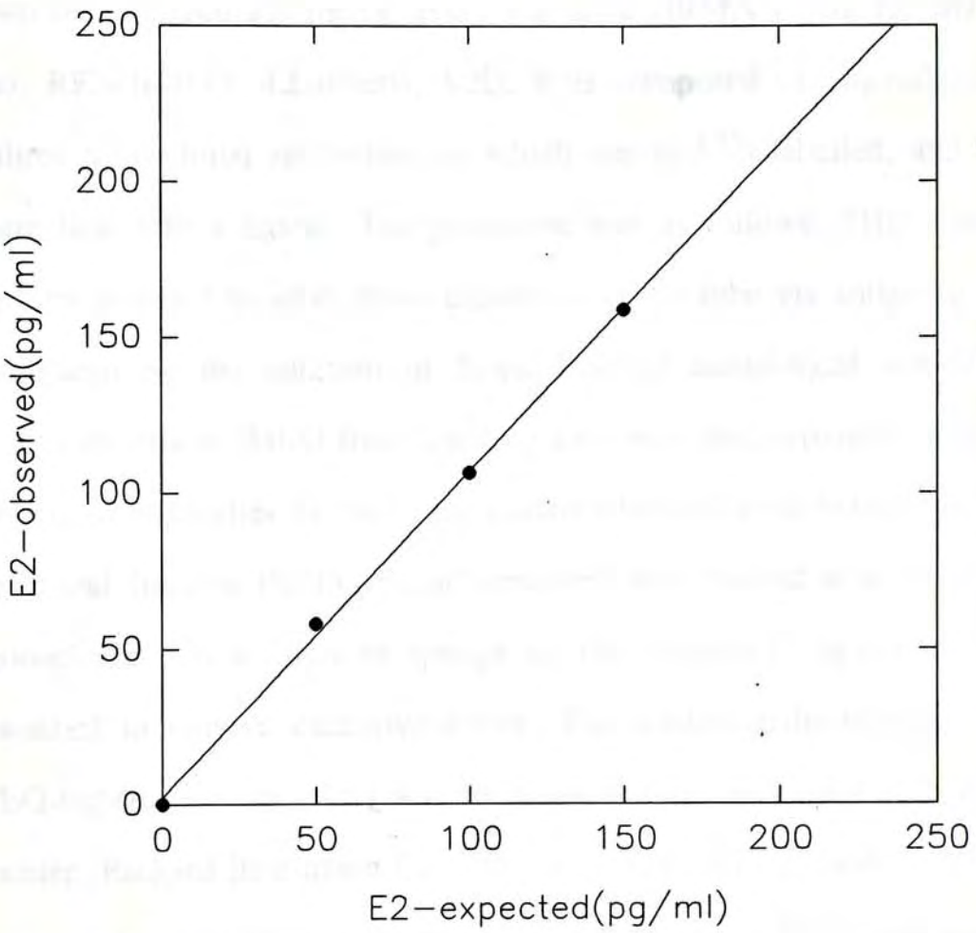

Fig. 9. The regression line of recovered vs expected value for E_2 which was assayed by "in-house" RIA.

Observed = 1.0085 × Expected + 6.783, r = 0.9997. Each [•] represents the mean of ten replicates.

and 1 ml of the organic phase was used for RIA, in duplicate.

There was a linear relationship between the measured E_2 concentration and the volume of diluted acid hydrolyzate used for extraction (**Fig.10**). The recovery of E_2 was 85%.

III.4. Determination of SHBG

A two-site immunoradiometric assay was used (IRMA Count for SHBG, DPC lot no. RKSHI-0173, LLanberis, UK). It is composed of ligand-coated tubes and three monoclonal antibodies, of which one is ^{125}I-labelled, and the other two are linked to a ligand. The procedure was as follows: SHBG in the sample was first attached to solid phase ligand coated on tube via antigenic site, and then captured by the addition of ligand-labelled monoclonal anti-SHBG antibodies. The excessive SHBG free-ligand on tube was then separated with the second monoclonal antibodies by the ligand-coated tube/antiligand bridge method. Finally the bound fraction (SHBG-ligand complex) was reacted with the ^{125}I-labelled monoclonals via a different epitope on the "captured" ligand, and the tube was washed to remove excessive tracer. The washed radio-labelled solid phase (SHBG-ligand-^{125}I complex) was then counted on Packard Cobra Auto-Gamma counter (Packard Instrument Co., Illinois, USA). All standards, controls, and samples were run together in the test, and the values of SHBG of samples were obtained by interpolation to standard curve.

III.5. Determination of urinary unconjugated cortisol

Urinary unconjugated cortisol was assayed by DPC RIA kit (DPC coat-a-count lot no. TK co5-519). The procedure included extraction of urinary steroids by dichloromethane, reconstitution of the extract with zero calibrator after evaporating off the organic solvent under a stream of nitrogen, and finally RIA as per the manufacturer's instructions. The solid phase of the ^{125}I-labelled antigen-

antibody complex was counted on a Packard Cobra Auto-Gamma counter.

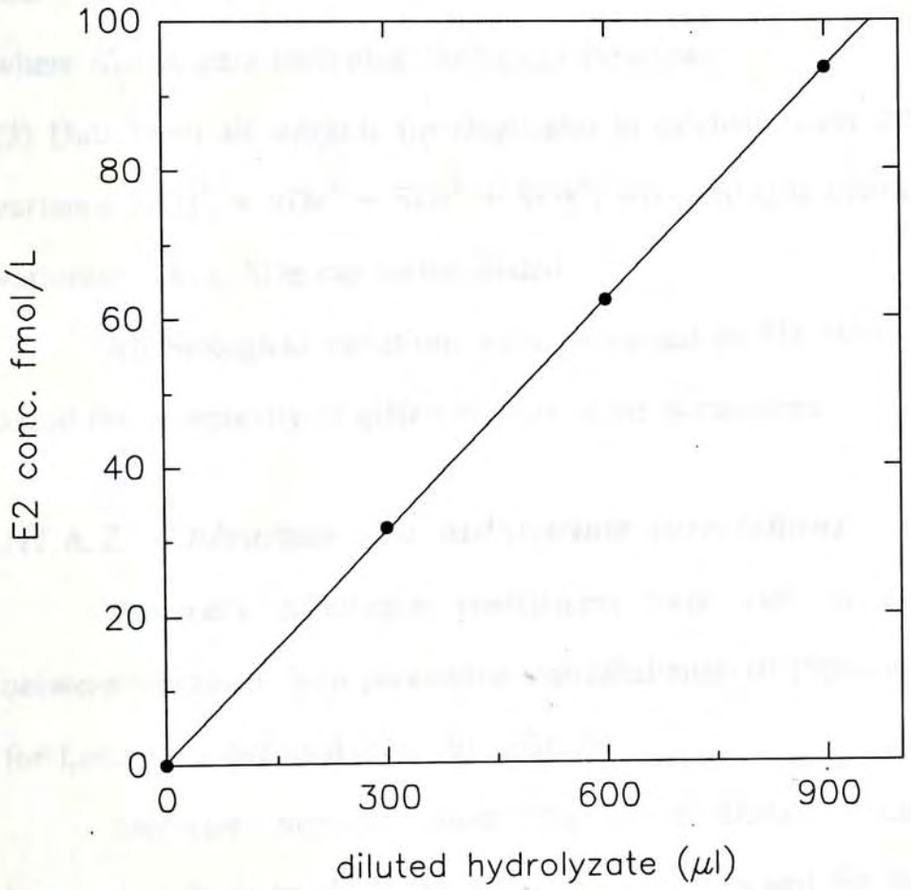

Fig.10. The dose-response curve of measured urinary total E2 vs various volumes of diluted acid hydrolyzate.

III.6. Statistical methods

III.6.1. Biological Variations

The components of biological variation were determined following the strategies proposed by Fraser (1986) which is based on the principle of nested analyses of variance (Fraser, 1989). There are three steps in this strategy:

(1) Analytical variance could be obtained by data from the duplicate analyses using the formula : SDa^2 = Sum of $(difference)^2/(2 \times$ no. of pairs). (2) For each individual, a single set of data is used to calculate the SD. The variance (SD^2) is made up of analytical and intraindividual variance. $SD^2 = SDa^2 + SDi^2$, where SDi is intra-individual biological variation.

(3) Data from all subjects (in singlicate) to calculate total SD ([SD]). The total variance $[SD]^2 = SDa^2 + SDi^2 + SDg^2$, where SDg is interindividual biological variation. Thus, SDg can be calculated.

All biological variations were expressed as SD/Mean × 100% (CV%) to avoid the complexity of different units of the parameters.

III.6.2. Univariate and multivariate correlations

Pearson's correlation coefficients were used to examine associations between variables. Non-parametric statistical analysis (Spearman Rank) were used for Lp(a) data due to skewed distribution.

Stepwise multiple linear regression analysis was performed with lipoprotein-lipids levels as the dependent variables and the independent variables including potential confounders, removed stepwise (probability for removal 0.05) to look at the interrelations between hormones and lipoprotein-lipids. Most of the experimental subjects were non-smokers (except one) and non-drinkers or drinkers taking small amount of beer or wine. These variables were not considered as possible confounders. There were three steps:

(a) Step 1. Linear regressions were performed with lipoprotein-lipid levels as the dependent variables and anthropometric parameters (BMI, WHR, and Age), serum sex hormones, urinary total and unconjugated sex hormones and SHBG as independent variables.

(b) Step 2. Calculated serum unbound sex hormone levels replaced serum total sex hormones in the regression, since there was a high degree of multicolinearity between them.

(c) Step 3. Triglyceride was added as one of the independent variables to see the relationship between HDL, its subfractions and sex hormones.

Chapter IV. Results

IV. 1. The characteristics of the experimental subjects and their lipoprotein-lipids profiles

The anthropometric characteristics of 46 apparently healthy Chinese men were summarized in **Table IV.1.A**. The values (Mean ± S.E.M.) for serum albumin, γ-glutamyl transferase (γGT) and urinary creatinine were also included in this Table.

Table IV.1.A. The anthropometric and biochemical characteristics of the experimental male subjects (n=46).

Parameter	Mean (SEM)	Actual Range
Age (yrs)	33 (1)	19--53
BMI (kg/m²)	22.6 (0.37)	17.56--30.2
Waist/Hip	0.85 (0.009)	0.72--1
Serum albumin (g/L)	47.4 (0.33)	42.4--52.5
γGT (U/L)	18.1 (2.06)	2.5--60
Urine creatinine (mmol/L)	13.65 (0.35)	8.58--19.34

As shown in **Table IV.1.B.** their lipoprotein-lipids expressed as the mean (±S.E.M.) values were: 4.79±0.15 mmol/L for cholesterol; 1.3±0.11 mmol/L for triglyceride; 2.9 ±0.14 mmol/L for LDL-C; 1.29±0.04 mmol/L for HDL-C; 0.38±0.031 mmol/L for HDL_2-C; and 0.92±0.023 mmol/L for HDL_3-C.

Expressed as mass concentrations, the values (mean ± S.E.M.) for apoA-I and apoB were 1.45±0.029 and 1.36±0.029 g/L, respectively. The actual range for Lp(a) is 55-868 mg/L with a mean value of 168.18 mg/L and the distribution of Lp(a) is highly skewed in this population.

Table IV.1.B. The lipoprotein-lipids profiles in 46 healthy Hong Kong Chinese men aged 19-53 yr.

Parameter	Mean (SEM)	Actual Range
Cholesterol (mmol/L)	4.79 (0.15)	2.8--7.0
Triglyceride (mmol/L)	1.3 (0.11)	0.49--3.40
LDL-C (mmol/L)	2.9 (0.14)	1.23--4.95
HDL-C (mmol/L)	1.29 (0.04)	0.84--2.02
HDL2-C (mmol/L)	0.38 (0.031)	0.13--0.96
HDL3-C (mmol/L)	0.92 (0.023)	0.63--1.25
Apo A-I (g/L)	1.45 (0.029)	1.06--1.84
Apo B (g/L)	1.36 (0.029)	0.98--1.79
Lp (a) [mg/L]	168.18 *	55--868

* Population showed a skewed distribution.

IV. 2. Levels of sex hormones in serum and urine, and urinary free cortisol

As shown in **Table IV.2**, the mean value for the excretion of urinary unconjugated testosterone was 1.39 nmol/d which is a very small proportion (about 1-2%) of urinary total testosterone (98 nmol/d). The mean value of urinary unconjugated estradiol was 142.8 pmol/d (< 1% of the total) and that of urinary total estradiol was 16.85 nmol/d. The value (mean±S.E.M.) for serum testosterone was 21±1.14 nmol/L, whereas that for serum estradiol was 139±4.65 pmol/L.

Since it is difficult to assay serum unbound sex steroids directly, their concentrations were calculated by the formula proposed by Södergád *et al.* (1982), using the association constants for steroid binding to SHBG and albumin. The formulae are shown in **Table IV.2.A and Table IV.2.B.** The calculated unbound testosterone and E_2 were in the range of 400-1560 pmol/L, and 2.21-

6.78 pmol/L respectively. When expressed in terms of percent of the total, the range for testosterone was 2.7-3.6 % and the range for E_2 was 2.97-3.02 %. It is noteworthy that the unbound fractions of testosterone and E_2 are quite similar, in terms of percentage, and with a very narrow range among the individuals.

With a range of 8.75 nmol/L to 46.75 nmol/L, the mean (±SEM) of values for SHBG concentration was 32.7±1.37 nmol/L in this group of Chinese men.

The urinary free cortisol in this population was in the range of 42 nmol/d to 262 nmol/d with a value (mean ±SEM) of 143±8.15 nmol/d.

Table IV.2. The sex hormones at serum and urinary levels and urinary free cortisol in 46 healthy Hong Kong Chinese men.

Parameter	Mean (SEM)	Actual Range
U-free testosterone (nmol/d)	1.39 (0.15)	0.59--6.49
U-total testosterone (nmol/d)	98 (7.8)	50--278
U-free-E_2 (pmol/d)	142.8(9.9)	45.3--328.8
U-total E_2 (nmol/d)	16.85 (0.81)	6.65--31.82
U-F-testo/U-total testo × 100 %	1.6 %	0.55--7.6%
U-F-E_2 / U-total E_2 × 100%	0.9%	0.27-1.72%
S-total testosterone (nmol/L)	21 (1.14)	12.6--57.5
S-free testosterone, calculated (nmol/L)	0.66 (0.03)	0.40--1.56
S-total E_2 (pmol/L)	139.2 (4.65)	74.4--224.5
S-free E_2, calculated (pmol/L)	4.15 (0.14)	2.21--6.78
SHBG (nmol/L)	32.2 (1.37)	8.75--55.5
U-free cortisol (nmol/d)	143 (8.15)	41.3--261.4

Table IV.2.A. Formula for the indirect calculation of unbound (free) testosterone levels in plasma ★

T = (Kt x Tf x S) ÷ (1 + Kt x Tf + KE x Ef + KD x Df + KAa x Aaf +KAe x Aef) + KA^1 x A x Tf +Tf

T = Total Testsosterone (mol/L)
Kt = Association constant for binding of Testosterone to SHBG
= $5.97 \times e^8$ M^{-1}
Tf = Free Testosterone (mol/L)
S = Sex Hormone Binding Globulin (mol/L)
KE = Association constant for binding of Estradiol to SHBG
= $3.14 \times e^8$ M^{-1}
Ef = Free Estradiol
= $4.80 \times e^{-12}$ mol/L
KD = Association Constant for binding of 5 alpha-Dihydrotestosterone to SHBG
= $10.7 \times e^8$ M^{-1}
Df = Free Dihydrotestosterone
= 67.1--351.0 x e^{-11} mol/L
KAa = Association Constant for binding of 5 alpha-androstane-3 alpha, 17ß-diol to SHBG
= $6.45 \times e^8$ M^{-1}
Aaf = Free 5 alpha-androstane-3 alpha, 17ß-diol
=33.5--80.7 x e^{-11} mol/L
KAe = Association Constant for binding of 5 alpha-androstene-3 alpha, 17ß-diol to SHBG
= $4.24 \times e^8$ M^{-1}
Aef = Free 5 alpha-androstene-3 alpha, 17ß-diol
= 200--585 x e^{-11} mol/L
KA^1 = Number of binding sites for Testosterone on each molecule of Albumin multiplied by the Association Constant for the binding of Testosterone to Albumin
= $4.06 \times e^4$ M^{-1}
A = Albumin Concentration (mol/L)+

\+ Molecular weight for serum albumin is 69,000

★ Södergård,R.; Bäckström, T.; Shanbhag, V.; and Carstènsen, H. (1982) Calculation of free and bound fractions of testosterone and estradiol-17ß to human plasma proteins at body temperature. J. Steroid Biochem. **16:**801-810

Table IV.2.B. Formula for the indirect calculation of unbound (free) 17ß-estradiol levels in plasma★

$$E2 = (KE \times Ef \times S) \div (1 + Kt \times Tf + KE \times Ef + KD \times Df + KAa \times Aaf + KAe \times Aef) + KA^2 \times A \times Ef + Ef$$

E2 = Total 17ß-estradiol (mol/L)
Kt = Association constant for binding of Testosterone to SHBG
= $5.97 \times e^8$ M^{-1}
Tf = Free Testosterone (mol/L)
S = Sex Hormone Binding Globulin (mol/L)
KE = Association constant for binding of Estradiol to SHBG
= $3.14 \times e^8$ M^{-1}
Ef = Free Estradiol (mol/L)
KD = Association Constant for binding of 5 alpha-Dihydrotestosterone to SHBG
= $10.7 \times e^8$ M^{-1}
Df = Free Dihydrotestosterone
= $67.1\text{--}351.0 \times e^{-11}$ mol/L
KAa = Association Constant for binding of 5 alpha-androstane-3 alpha, 17ß-diol to SHBG
= $6.45 \times e^8$ M^{-1}
Aaf = Free 5 alpha-androstane-3 alpha, 17ß-diol
$=33.5\text{--}80.7 \times e^{-11}$ mol/L
KAe = Association Constant for binding of 5 alpha-androstene-3 alpha, 17ß-diol to SHBG
= $4.24 \times e^8$ M^{-1}
Aef = Free 5 alpha-androstene-3 alpha, 17ß-diol
= $200\text{--}585 \times e^{-11}$ mol/L
KA^2 = Number of binding sites for 17ß-estradiol on each molecule of Albumin multiplied by the Association Constant for the binding of 17ß-estradiol to Albumin
= $4.21 \times e^4$ M^{-1}
A = Albumin Concentration (mol/L)+

+ Molecular weight for serum albumin is 69,000

★ Södergård,R.; Bäckström, T.; Shanbhag, V.; and Carstènsen, H. (1982) Calculation of free and bound fractions of testosterone and estradiol-17ß to human plasma proteins at body temperature. J. Steroid Biochem. **16**:801-810

IV. 3. Biological variations

Table IV.3. is a summary of the biological variations of serum lipoprotein-lipids, serum and urinary sex hormones and cortisol in 46 healthy Chinese men. **Table IV.3.A** and **Table IV.3.B** show the correlation of serum lipoprotein-lipids, sex hormones, as well as urinary sex hormones between samples taken approximately three weeks apart.

Table IV.3. The biological variations* of serum lipoprotein-lipids, serum sex hormones and urinary sex hormones and cortisol in 46 healthy Hong Kong Chinese men aged 19-53 years

Variables	No. of subjects	CVa (%)	CVi (%)	CVg (%)	Index of individuality (CVi/CVg)
Lipoprotein-lipids					
Triglyceride	46	1.7	23.9	34.9	0.7
Cholesterol	46	2.3	6.3	20.8	0.3
Lp(a)	46	5.5	14.3	102	0.14
HDL	46	3.8	5.7	21.7	0.3
HDL2	46	11.4	14.8	53	0.3
HDL3	46	4.1	6.1	15.1	0.4
apoA-I	46	1.8	4	12.7	0.3
apoB	46	1.2	4.7	13.4	0.4
Hormones					
S-E2	46	6.6	23.4	8.7	2.7
S-Testosterone	46	4.1	9.2	35.4	0.3
U-total E2	46	4.9	30.4	16.85	1.8
U-free E2	46	7.1	38.5	26.1	1.5
U-total Testo	46	7.2	25	47.3	0.5
U-free Testo	46	5.5	51.7	61.4	0.8
U-free Cortisol	46	7.8	19.7	27.7	0.7

CVa=analytical variation as coefficient of variation
CVi=intra-individual variation as coefficient of variation
CVg=inter-individual variation as coefficient of variation
* The components of variation were calculated after Fraser (1986), with untransformed data.

Table IV.3.A. Correlations of serum lipoprotein-lipids between short-term (3-week) variations in 46 healthy Hong Kong Chinese men

Parameters	r	p
Triglyceride	0.75	<0.0001
Cholesterol	0.89	<0.0001
apoA-I	0.88	<0.0001
apoB	0.94	<0.0001
HDL-total	0.90	<0.0001
HDL2	0.88	<0.0001
HDL3	0.80	<0.0001
Lp(a)	0.98	<0.0001

Table IV.3.B. Correlations of serum and urinary sex hormones and urinary unconjugated cortisol between short-term (3-week) variations in 46 healthy Hong Kong Chinese men

parameters	r	p
S-testosterone	0.9	<0.0001
S-17ß estradiol	0.15	NS
U-total E2	0.36	0.015
U-unconjugated E2	0.36	0.015
U-total testosterone	0.82	<0.0001
U-unconj. testosterone	0.48	0.001
U-unconj. cortisol	0.6	<0.0001

The subjects were asked not to change their life style to try to minimize pre-analytical sources of variation. All blood samples were collected sitting between 8:30 am to 10:00 am. Among variables serum testosterone showed good agreement between short-term repeated samples (r=0.9), while serum E_2 did not show any significant correlation (**Table IV.3.B.**) implying greater biological variability. The greater correlation between duplicates for urinary estradiol (r=0.36, p=0.015) than for serum estradiol (r=0.15, NS) suggests that the use of urinary estradiol as an index of estradiol production could be more useful. However the intraindividual variability of 17 β-E_2 in serum is greater than that of serum testosterone (CVi = 23.4, 9.2 respectively) (**Table IV.3**). and therefore, repeated measurement of E_2 would seem to be preferred.

The index of individuality was calculated as CVi/CVg (Fraser, 1986). Lp(a) had the least index of individuality (0.14), while serum E_2, urinary total E_2 and urinary unconjugated E_2 had the largest indices of individuality (2.7, 1.8, 1.5, respectively) among all assayed parameters.

For lipoprotein-lipids, all show good agreement between these two occasions (Table **IV.3.A**). Lp(a) and apoB appear to be the most stable in this population (r=0.98, 0.94 respectively), whereas triglyceride shows the greatest short-term variation (r=0.75). Cholesterol, HDL-C, and HDL subfractions--HDL_3-C and HDL_2-C, as well as apoA-I are in between with correlation coefficients of 0.89, 0.9, 0.88, 0.80, 0.88 respectively.

It is noteworthy that the HDL_3-C has less intra- and inter- individual variations than those of HDL_2-C.

IV. 4. Univariate correlation

The scatter graphs for univariate correlation are compiled in **Appendix ii.** The univariate correlation matrix is summarized in **Table IV.4**. The results are as follows:

Table IV.5. Univariate correlation matrix of sex hormones and lipoprotein-lipids based on data of 46 apparently healthy Chinese men

	Ef	Tf	fuTes	tuTes	s-Tes	fu-E2	tu-E2	s-E2	ufCort	Chol	Trig	LDL	HDL	HDL 3	HDL 2	Apo A-I	Apo B	SHBG	Lp(a)	Age	BMI	WHR	γGT	rHDL 2/3	rHDL 3/2
Ef	-	↑ +	↑ ***	↑	↑ +	↑ *	↑	↑ +	↑	↓ ± *	↓ *	↓ ± *	↑	↑	↑ *	↑	↓ ±0.07	↑	↑	↓ *	↓	↓	↓	↑ ± *	↓
Tf	-	-	↑ ****	↓	↑ +	↑	↑	↑ +	↑	↓	↓ ***	↓	↑ ***	↑	↑ ****	↑ *	↓	↑ +	↓	↓	↓ ***	↓	↓ **	↑ ****	↓ ***
fuTes	-	-	-	↑	↑ +	↑ ****	↑ *	↑ ***	↑ **	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↓ *	↓	↓	↓	↑	↓
tuTes	-	-	-	-	↓	↑	↑ +	↑	↑ *	↓	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑
s-Tes	-	-	-	-	-	↑	↑	↑ +	↑	↓	↓ ***	↓	↑ ***	↑	↑ ****	↑ *	↓	↓ ****	↓	↓ *	↑ ***	↓	↓ *	↑ ***	↓ ***
fu-E2	-	-	-	-	-	-	↑ *	↑ *	↑	↑	↓	↑	↑	↑ *	↓	↑	↑	↓	↑ *	↑	↑	↑	↑	↓	↑
tu-E2	-	-	-	-	-	-	-	↑	↑ ± *	↓ *	↓	↓ *	↓	↓	↑	↓	↓ **	↓	↓	↓	↑	↑	↑	↑	↓
s-E2	-	-	-	-	-	-	-	-	↑	↓ *	↓ *	↓ ± *	↑	↑	↑ *	↑	↓ ±0.06	↑	↑	↓	↓ *	↓	↓	↑	↓
ufCort	-	-	-	-	-	-	-	-	-	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↓	↓	↑	↓
Chol	-	-	-	-	-	-	-	-	-	-	↑ *	↑ +	↑	↑ ****	↓	↑ * *	↑ +	↑	↑	↑ *	↑ **	↑ *	↑ ***	↓ *	↑ *
Trig	-	-	-	-	-	-	-	-	-	-	-	↑	↓ ***	↓	↓ ****	↓	↑ *	↓ ***	↓	↑	↑ +	↑ *	↑ ***	↓ ***	↑ **
LDL	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑ ***	↓0.06	↑	↑ +	↑	↑ *	↑ *	↑ *	↑ *	↑ ***	↓ ***	↑ **
HDL	-	-	-	-	-	-	-	-	-	-	-	-	-	↑ +	↑ +	↑ +	↑	↑ *	↑	↓	↓ **	↓	↓ *	↑ +	↓ +
HDL3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑ +	↑ ****	↑	↑ ±0.07	↑	↓	↑	↑	↓	↑
HDL2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑ +	↓	↑ **	↓	↓	↓ +	↓ *	↓ ***	↑ +	↓ +
ApoA¹	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑ ****	↑ *	↑	↓	↓	↓	↓	↑ ***	↓ ***
ApoB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑ ±0.09	↑	↑	↑	↑ *	↓	↑
SHBG	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↑	↓ *	↑	↓	↑ **	↓ *
Lp(a)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑	↓	↑	↓	↑
Age	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑ *	↑ ****	↑ ***	↓	↑ **
BMI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑ +	↑ ***	↓ +	↑ +
WHR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑ ***	↓ *	↑ ***
γGT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓ ***	↑ ****
rHDL 2/3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓ +
rHDL 3/2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

↑/↓ significantly direct/reverse correlation; ↑/↓ not significantly direct/reverse correlation

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.0005$; ***$p \leq 0.001$; ****$p = 0.0001$; +$p < 0.0001$; ± p is on border line

Abbreviations: Ef=free E_2 calculated; Tf=free testosterone calculated; fuTes=urinary free testosterone; tuTes=urinary total testosterone; s-Tes=serum total testosterone; fu-E_2=urinary free E_2; tu-E_2=urinary total E_2; s-E_2=serum total E_2; ufCort=urinary free cortisol; Chol=total cholesterol; Trig=triglyceride; LDL=low density lipoprotein; HDL=high density lipoprotein; HDL_2, HDL_3= high density lipoprotein subfraction 2 and 3; ApoA-I=apolipoprotein A-I; ApoB=apolipoprotein B; SHBG=sex hormone binding globulin; Lp(a)=lipoprotein (a); BMI=body mass index; WHR=waist and hip ratio; γGT= gamma glutamyl transpeptidase; rHDL2/3, rHDL3/2= the ratios of HDL_2 and HDL_3.

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IV.4.1. Inter-relationship among serum and urinary sex hormones

The urinary *non-conjugated* E_2 was significantly correlated with both urinary total E_2 ($r=0.353$, $p=0.016$) and serum total E_2 ($r=0.325$, $p=0.027$). Neither urinary total E_2 nor urinary total testosterone was correlated with serum total estradiol and testosterone. Urinary non-*conjugated* E_2 and testosterone were significantly correlated with serum total estradiol and testosterone, respectively, with $r=0.325$, $p=0.0274$ and $r=0.596$, $p<0.0001$ (**Appendix ii.f--ii.g**).

The serum E_2 was significantly correlated with the serum testosterone with $r=0.57530$, $p<0.0001$. (**Appendix ii.g**). However, urinary total testosterone was not correlated with urinary non-conjugated testosterone.

IV.4.2. Urinary free cortisol and sex hormones and serum lipoprotein-lipids

Interestingly the urinary free cortisol is positively correlated with both urinary total and unconjugated testosterone ($r=0.317$, $p=0.032$; $r=0.397$, $p=0.0062$, respectively) (**Appendix ii.g**) but neither correlated with serum testosterone and estradiol nor correlated with urinary total and unconjugated estradiol. However, urinary free cortisol concentrations showed no significant associations with any lipoprotein variable. If this is not due to chance, an explanation is not immediately apparent.

IV. 4.3. Correlation between urinary sex hormones and serum lipoprotein-lipids

As shown in **Table IV.4 and Appendix ii.f**, the urinary unconjugated E_2 excretion is directly but weakly associated with the serum levels of Lp(a) ($r=0.3662$, $p=0.0123$) (for untransformed data only) and HDL_3-C ($r=0.3073$, $p=0.038$), and this association is even stronger in terms of percent urinary unconjugated E_2 excretion {with $r=0.387$, $p=0.008$ (for untransformed data

and total serum testosterone show a weak *direct* relationship with HDL, HDL_2, apoA-I, and SHBG and a stronger *inverse relationship* with triglyceride. **(Appendix ii.o.)**. Unbound and total serum E_2 are inversely and weakly correlated with cholesterol, triglyceride, and LDL and directly with HDL_2 again weakly, but not with HDL and apoA-I. **(Appendix ii.m.)**.

IV. 4.6. Correlations between anthropometric variables, sex hormones and lipoprotein-lipids

Age is positively correlated with cholesterol ($r=0.339$, $p=0.021$) and LDL-C ($r=0.352$, $p=0.0166$) **(Appendix ii.c.)** and inversely with the calculated serum unbound E_2 ($r=-0.340$, $p=0.0209$), serum total testosterone ($r=-0.309$, $p=0.0364$), and urinary un-conjugated testosterone ($r=-0.337$, $p=0.0218$). **(See Appendix ii.a.)**. No correlation is found with the urinary E_2 level. Age is directly associated with BMI ($r=0.293$, $p=0.0478$), WHR ($r=0.508$, $p=0.0003$) (Appendix ii.d.), and γGT ($r=0.41128$, $p=0.0045$) **(Appendix ii.b.)**.

BMI is positively correlated with cholesterol ($r=0.397$, $p=0.0063$), triglyceride ($r=0.589$, $p<0.0001$), LDL-C ($r=0.372$, $p=0.0109$) **(Appendix ii.c.)**, WHR ($r=0.564$, $p<0.0001$), γGT ($r=0.442$, $p=0.0021$), and age **(Appendix ii.d)**. An inverse correlation is found between BMI and the calculated serum unbound and the serum total testosterone ($r=-0.42454$, $p=0.0033$ and $r=-0.407$, $p=0.0050$, respectively) **(Appendix ii.d.)**, HDL ($r=-0.402$, $p=0.0056$), HDL_2 ($r=-0.543$, $p<0.0001$), HDL_2/HDL_3 ($r=-0.570$, $p<0.0001$) **(Appendix ii.c.)** and SHBG ($r=-0.32947$, $p=0.0254$) **(Appendix ii.d)**.

WHR is directly associated with cholesterol ($r=0.336$, $p=0.0226$), triglyceride ($r=0.325$, $p=0.0277$), LDL-C ($r=0.343$, $p=0.0195$) **(Appendix ii.r.)**, γGT ($r=0.420$, $p=0.0037$) and age ($r=0.50787$, $p=0.0003$) **(Appendix ii.d.)**, and inversely with HDL_2-C ($r=-0.356$, $p=0.015$) and HDL_2/HDL_3

(r=-0.370, p=0.012) **(Appendix ii.r.)**. No association was found with any serum or urinary sex hormone variable.

γGT, interestingly, is positively correlated with all anthropometric variables (BMI, WHR, and Age) (r=0.442, p=0.0021; r=0.420, p=0.0037; r=0.411, p=0.0045, respectively) **(Appendix ii.b. & Appendix ii.d.)** and with all atherogenic lipoprotein-lipids (Cholesterol, Triglyceride, LDL-C, and ApoB) (r=0.448, p=0.0018; r=0.474, p=0.0009; r=0.441, p=0.0022; r=0.291, p=0.0501, respectively) **(Appendix ii.h.)**, but inversely with the serum testosterone (both the unbound and the total) (r=-0.391, p=0.0072 and r=-0.364, p=0.0128, respectively) and the anti-atherogenic lipoprotein-lipids HDL and HDL_2 and HDL_2/HDL_3, with r=-0.310, p=0.0361; r=-0.449, p=0.0018; and r=-0.461, p=0.0012, respectively **(Appendix ii.h)**.

SHBG is positively associated with serum testosterone (r=0.516, p=0.0002) **(Appendix ii.o.)**, HDL-C (r=0.373, p=0.0107), and HDL_2-C (r=0.398, p=0.0062) and inversely associated with triglyceride (r=-0.436, p=0.0025) **(Appendix ii.n.)** and BMI (r=-0.330, p=0.0254) **(Appendix ii.d.)**.

IV. 4.7. Correlation of the ratio of HDL_2 and HDL_3 and other variables

HDL_2/HDL_3 is directly correlated with the serum testosterone (both unbound and total) (r=0.504, p=0.0004; r=0.466, p=0.0011, respectively) **(Appendix ii.o.)**, and SHBG (r=0.379, p=0.0095) **(Appendix ii.n.)**.

The ratio is inversely correlated with cholesterol (r=-0.325, p=0.0275) **(Appendix ii.e.)**, LDL-C (r=-0.416, p=0.004) **(Appendix ii.k.)**, triglyceride (r=-0.489, p=0.0006) **(Appendix. ii.p.)**. It is also associated with BMI (r=-0.570, p<0.0001) **(Appendix ii.c.)**, WHR (r=-0.370, p=0.012) **(Appendix ii.r.)**, and γGT (r=-0.461, p=0.0012) **(Appendix ii.h.)**. The correlations with total HDL (r=0.676, p<0.0001) **(Appendix ii.i.)**, apoA-I

(r=0.427, p=0.0031) would be expected.

IV. 5. Multiple linear stepwise regression

Table IV.5. Stepwise multiple linear regression of lipoprotein-lipids* (dependent variable) on BMI, W/H Ratio, and Age (independent variables) in 46 healthy Chinese men aged 19-53 years.
t and p values are indicated.

Dependent variable → / In-dependent variable ↓	Chol	Trig	LDL	HDL	HDL2	HDL_2 / HDL_3	ApoAI ApoB HDL_3 Lp(a)
BMI (*t*)	+2.870	+4.829	+2.660	-2.913	-4.285	-4.602	-
(*p*)	0.0063	<0.0001	0.0109	0.0056	<0.0001	<0.0001	
WHR / Age	-	-	-	-	-	-	-

* All data were not transformed

The initial result from multiple linear regression of lipoprotein-lipids (dependent variable) on the anthropometric variables BMI, WHR, and age (**Table IV.5**) indicates that BMI is directly and significantly associated with cholesterol (t=+2.8698, p=0.0063), triglyceride (t=+4.8289, p<0.0001), and less strongly with LDL-C with p=0.0109. It is also inversely associated with HDL-C (t=-2.9127, p=0.0056), HDL_2-C (t=-4.2853, p<0.0001), and the ratio of HDL_2-C and HDL_3-C with t=-4.602, p<0.0001. No association is found with apoA-I, apoB, Lp(a) and HDL_3-C. Neither age nor WHR in this population has significant correlation with lipoprotein-lipids in the model.

After adjustment for BMI, WHR, Age, SHBG, and sex hormones (serum and urinary levels), as shown in the **Table IV.5.A,** the levels of HDL3, apoB, and untransformed Lp(a) are directly (t=+2.8, p=0.0076; t=+2.32, p=0.025; t=+2.6, p=0.0123, respectively) whereas the level of triglyceride (t=-2.06, p=0.046) inversely correlated with the urinary excretion of unconjugated estradiol. The urinary total E_2 excretion is inversely correlated with cholesterol (t=-2.60, p=0.0126), LDL-C (t=-2.53, p=0.0153), HDL_3 (t=-2.05, p=0.0468), and apoB (t=-3.06, p=0.0038). **BMI** remained strongly positively correlated with cholesterol (t=+3.23, p=0.0024), triglyceride (t=+4.46, p<0.0001), LDL-C (t=+2.98, p=0.0046), and inversely with HDL_2 (t=-3.10, p=0.0034).

HDL-C is directly associated with serum total testosterone (t=+3.16, p=0.0028) and serum 'free' testosterone (t=+3.44, p=0.0013).

Triglyceride is not significantly associated with testosterone when adjusted for SHBG and anthropometric confounders including BMI, but reaches borderline significance with the urinary unconjugated E_2 excretion (t=-2.06, p=0.046). However, it is strongly, persistently, and directly associated with BMI (t=+4.455, p<0.0001)and inversely associated with SHBG (t=-2.6, p=0.014).

Furthermore, since the metabolism of total HDL and its subfractions associates closely with triglyceride concentrations (Patsch ***et al.,*** 1983, 1987; Silverman ***et al.,*** 1993), triglyceride was also included in the model for the relationship of HDL-C and sex hormones. As shown in **Table IV.5.B,** after triglyceride concentration was included in the model, the relationship between urinary E_2 (both total and non-conjugated) and HDL_3-C did not change, as did not the associations between serum testosterone (both total and unbound) with HDL_2-C (t=+2.60, p=0.0128 and t=+2.90, p=0.0052, respectively). However, the association of BMI with HDL seen univariately was abolished after

Table IV.5.A. Stepwise multiple linear regression of lipoprotein-lipids (dependent variable) on BMI, W/H Ratio, Age, SHBG, and serum and urinary Sex Hormones (independent variables) in 46 healthy Hong Kong Chinese men aged 19-53 years.*
t and p values are indicated.

Dependent variable → In-dependent variable ↓	Chol	Trig	LDL	HDL	HDL2	HDL3	Apo A-I	Apo B	Lp(a)§
FU-E2 (*t*)	-	-2.06				+2.8		+2.32	+2.6
(*p*)		0.046	-	-	-	0.0076	-	0.025	0.0123
TU-E2 (*t*)	-2.60		-2.53			-2.05		-3.06	
(*p*)	0.0126	-	0.0153	-	-	0.0468	-	0.0038	-
S-F-E2 (*t*)	-	-	-	-	-	-	-	-2.04	-
(*p*)								0.0477	
S-Test (*t*)	-			+3.16	+2.60				
(*p*)		-	-	0.0028	0.0128	-	-	-	-
S-F-Testo (*t*)				+3.44	+2.90				
(*p*)	-	-	-	0.0013	0.0052	-	-	-	-
BMI (*t*)	+3.23	+4.455	+2.98		-3.10				
(*p*)	0.0024	<0.0001	0.0046	-	0.0034	-	-	-	-
SHBG (*t*)		-2.6					+2.37		
(*p*)	-	0.014	-	-	-	-	0.02	-	-

* All data were not transformed

§ When transformed Lp(a) concentrations were added in the place of raw skewed data the positive association with urinary nuconjugated E_2 was no longer significant.

Table IV.5.B. Stepwise multiple linear regression of lipoprotein-lipids (dependent variable) on BMI, W/H Ratio, Age, SHBG,* **Triglyceride** *and serum and urinary Sex Hormones (independent variables) in 46 healthy Hong Kong Chinese men aged 19-53 years.* **t** *and* **p** *values are indicated*

Dependent variable → / In-dependent variable ↓	HDL	HDL2	Apo A-I	HDL3
FU-E2 (*t*)	-	-	-	+2.8
(*p*)				0.0076
TU-E2 (*t*)	-	-	-	-2.05
(*p*)				0.0468
S-F-E2	-	-	-	-
S-Test (*t*)	-	+2.60	-	-
(*p*)		0.0128		
S-F-Testo (*t*)	+2.12	+2.94	-	-
(*p*)	0.0401	0.0052		
BMI (*t*)	-	-3.10	-	-
(*p*)		0.0034		
TU-testo	-	-	-	-
Trig (*t*)	-3.68	-	-	-
(*p*)	0.0006			
SHBG (*t*)	-	-	+2.37	-
(*p*)			0.02	

* All were un-transformed raw data

Chapter V. Discussion

V. 1. Experimental subjects and their lipoprotein-lipids profiles

As summarized in **Table IV.1.B** , the mean (±S.E.M.) values of lipoprotein-lipids in 46 apparently healthy Chinese men were all in the range typical of normal western men (Martini ***et al.,*** 1984). HDL_3-C level in men was higher than that of HDL_2-C. That is in line with the typical pattern documented in the literature (Anderson ***et al.,*** 1978; Martini ***et al.,*** 1984; Atger ***et al.,*** 1990). It is noteworthy that there is no difference in values for these lipoprotein-lipids profiles between the Caucasian and the Hong Kong Chinese. The total variation expressed as CV% in HDL_2-C is 56.2 which is much higher than that for HDL_3-C (16.8 %), i.e. HDL_2-C is more variable between individuals than HDL_3-C. The values (Mean±S.E.M.) for apoA-I and apoB were 1.45±0.029 and 1.36±0.029 g/L, respectively, and are higher than those reported by Masarei ***et al.*** (1986) (1.31±0.03 and 1.11±0.05 g/L). Different specificity of the antiserum and the standardization of the method might have accounted for the slight difference in values. The range of apoA-I concentration of our present study (1.06-1.84 g/L) appears to be close to that reported by Chambless ***et al.*** (1992) (0.96-1.72 g/L). However, it must be pointed out that the latter was obtained by combining values of 17 males and 23 females. Lp(a) concentrations (55-868 mg/L) are in the same order as those (20-750 mg/L) reported by Sandholzer[1] ***et al.*** (1992) among Singapore Chinese. Although there is considerable variation between individuals in this population, these two populations of Chinese individuals appear to have comparable levels. Lp(a) assays are not well standardized and therefore comparison of populations is difficult. As shown in **Table IV.3**, HDL_3-C has less intra- and inter- individual

variations than those of HDL_2-C. It has been documented that variations in HDL-C concentration both between and within subjects are mostly due to changes in subfraction HDL_2-C, and the level of HDL_3-C in men is rather constant (Anderson, 1978, Wood & Haskell, 1979; Puchois ***et al.,*** 1987). The value of HDL_2-C was obtained by subtraction of HDL_3-C from HDL-C, therefore greater analytical variation might be expected. From the values of CVi and CVa, obviously, improvement in determination of HDL-C and its subfractions should be sought, since CVa $>1/2$ CVi (Fraser, 1986).

Lp(a) has the least index of individuality (0.14). The very low index of individuality of Lp(a) is a reflection of the small change between short-term repeated samples. When the index of individuality is low, particularly less than 0.6, the variable has a relatively wide reference range with respect to the range of intraindividual variation, and the conventional reference values are of little help in making decisions about the probability of a result representing a 'normal' value for that individual (Fraser, 1986). The index of individuality of the serum lipoprotein-lipids is generally low. However it has become accepted practice to specify upper or lower 'desirable' values based upon risk data from population studies.

The correlation coefficient between HDL and apoA-I ($r=0.859$) is in agreement with that of Väisänen ***et al.***(1992) ($r=0.803$), and of Levinson & Wagner (1992) ($r=0.874$). Also apoB, the protein moiety of LDL-C is strongly correlated with LDL-C and the correlation coefficient of $r=0.856$ is in agreement with that of Väisänen ***et al.*** ($r=0.856$). ApoB is also strongly correlated with total cholesterol with a corrlation coefficient in agreement with that of Levinson & Wagner ($r=0.918$ in this experiment, and $r=0.866$ for the latter). As expected, HDL-C and HDL_2-C are correlated inversely with triglyceride. Since HDL-C is strongly positively associated with HDL_2-C, and HDL_2 is involved in

the clearance of triglyceride as documented (Anderson, 1978; Fielding and Fielding, 1981; Patsch *et al.,* 1983, 1987, 1992; Martini *et al.,* 1984; Silverman *et al.,* 1993), the metabolism of HDL-C is associated closely with triglyceride.

HDL_3 is directly correlated with LDL-C ($r=0.450$, $p=0.0017$) and apoB ($r=0.51927$, $p=0.0002$) and the association with the latter is even stronger than the former. This may imply that there is some metabolic connection between HDL_3 and apoB, since the cholesterol-lowering drug nicotinic acid also decreases HDL_3 levels (Blum *et al.,* 1977; Shepherd *et al.,* 1979).

The untransformed Lp(a) does not correlate with any lipoprotein-lipids except, weakly but significantly with LDL-C ($r=0.3$, $p=0.028$). A possible explanation is that the present experimental protocol for estimating LDL-C actually includes Lp(a)-cholesterol in the value for LDL-C as Lp(a) is precipitated by $MgCl_2$-dextran sulfate during the procedure for measurement of HDL-C, the calculated value for LDL-C actually represents non-HDL-cholesterol which contains both LDL and Lp(a). After subtracting the Lp(a) value from that of the calculated LDL-C, the *true* LDL-C value and Lp(a) are not correlated. Similar results have also been reported in a study when heparin-manganese was used as a precipitating agent for separation of HDL-C and LDL-C, and the Friedewald formula was used for calculation (Dahlen *et al.,* 1986).

V. 2. Levels of sex hormones in serum and urine

The values of sex steroids in 46 healthy Chinese men are compared with others cited in literature as shown in **Table V.2.** It seems that the present results are comparable. In terms of concentrations both unbound fractions are highly correlated with serum total levels ($r > 0.99$), possibly, at least partly, due to the fact that total testosterone and total estradiol are factors in calculation of both variables, and the correlation may be partly spurious. It also suggests they are giving similar information.

Table V.2. Values of sex steroids in 46 healthy Hong Kong Chinese men (aged 19-53 years) compared to others cited in literature

Variable	Chinese (n=46)	Others	✸ Ref.
U-f -Testo (mean±SEM)	1.39±0.15	1.43 (median)	1
		0.65---3.16	1
(range) nmol/d	0.59---6.49	0.18---7.08	1a
		1.34 (mean)	1b
U-T-Testo (mean±SEM)	98±7.8	121.5---399.3	2
(range) nmol/d	50---278	173.5---468.5 (20-50yr)	3
		138.8---208.2 (> 50yr)	3
		204.9	4
		291.7	5
U-f-E2 (mean±SD)	142.8±67	200±130	
(range) pmol/d	45.3---328.8		6
U-T-E2 (mean±SD)	16.85±5.5		
(range) nmol/d	6.65---31.82	0-22	3
S-T-Testo (mean±SD)	21±7.7	25.2±8.7 (n=243,36-61yr)	2
		13.7 (n=98, 52-74yr)(Japanese)	7
		19.85±4.68	3
(range) nmol/L	12.6---57.5	10.42---29.5 (n=14, 20-28yr)	8
S-T-E2 (mean±SD)	139.2±31.5		
(range) pmol/L	74.4---224.5	110-264	10
		29-132	3
		ND-161.5	9
S-F-E2, calculated* (mean ± SD)	4.15±0.95		
(range) pmol/L	2.21---6.78		
% of total (mean±SD)	(2.98±9.7e^{-5}) %		
(range) %	[2.97---3.02] %	[1.5-3.8] %	*
S-F-testosterone, calculated* (mean±SD)	0.66±0.21		
(range) nmol/L	0.40---1.56		
% of total (mean±SD)	(3.2±0.17) %		
(range) %	(2.7---3.6) %	(0.9-5.5) %	*

* by the method of Södergård **et al.** (1982) J. Steroid Biochem. 16:801-810
✸references listed under appendix iii.

The present results are within the ranges reported in the literature as reviewed by Södergád (1982). However, the urinary unconjugated testosterone (with the value of 0.6-7.6% of the total) and estradiol (with the value of 0.27-1.7% of the total) have very wide ranges of variation between the individuals. This probably indicates that there are great variations between individuals in terms of production and metabolic clearance rates.

As shown in **Table IV.2**, the excretion of urinary un-conjugated sex hormones is of a very small proportion (about 1-2%) of the urinary total sex hormones. This indicates that most of the sex hormones are excreted as conjugated metabolites rather than in an unmetabolized form.

SHBG, with a range of 8.75 nmol/L to 46.75 nmol/L and a coefficient of variation about 29% among the population, is significantly affected by BMI with a Pearson's correlation coefficient of -0.33 ($p=0.0254$), but not by age and WHR. The mean (±SEM) of values for SHBG concentration is 32.7±1.37 nmol/L in this group of Chinese men, and 20.02±2.9 nmol/L in a group of Caucasian men reported by Peiris ***et al.*** (1993). BMI, WHR and age are said to be factors affecting SHBG concentration. The values for BMI, and WHR were similar in the two groups, although the age in Chinese men tended to be older (SHBG levels increase with age). We can not be sure that the assays were similarly calibrated, but it is possible Chinese men have higher values for SHBG. Nevertheless, both groups of healthy male subjects have lower values than healthy women (the range of mean values is 39.7-45 nmol/L in females aged 20-69 years) (Preziosi ***et al.,*** 1993).

As shown in **Table IV.3.B** serum testosterone showed good agreement between short-term repeated samples ($r=0.9$). This finding is in agreement with the view that plasma testosterone level in men is fairly constant (Lipsett ***et al.,*** 1968). It also lends support to the suggestion that measurement of plasma

testosterone in men by using only a single morning blood sample is satisfactory and convenient for epidemiological purposes (Dai ***et al.,*** 1981). However the greater intraindividual variability of 17 β-E_2 (**Table IV.3**) in serum suggests that the repeated measurement of E_2 in men would seem to be preferred. Serum E_2, urinary total and unconjugated E_2 have the largest indices of individuality (2.7, 1.8, 1.5, respectively) among all assayed parameters. When the index of individuality is high, particularly greater than 1.4, the population reference range is of more use (Fraser, 1986).

The results showed that urinary *non-conjugated* E_2 was significantly correlated with both urinary total E_2 and serum total E_2. Since only a very small amount of E_2 is derived from extraglandular transformation of androstenedione and most E_2 in men is directly secreted or transformed from the secreted testostosterone by aromatization (Bardin, 1986), the measurement of the urinary E_2 both in conjugated and/or non-conjugated forms would be expected to reflect the production and/or secretion rate of E_2. The results also showed that urinary non-conjugated E_2 and testosterone were significantly correlated with serum total testosterone and estradiol, but that both the urinary total E_2 and testosterone were not correlated with serum total testosterone and estradiol. Since most of the serum testosterone in males is secreted by the testes (Lipsett ***et al.,*** 1968), the correlation between urinary non-conjugated testosterone and serum testosterone concentration implies that its secretion rate is likely to be a determinant of urinary non-conjugated testosterone excretion. By the same token, the secretion rate is probably a determinant of urinary non-conjugated E_2 excretion, although most serum E_2 derives from the transformation of serum testosterone rather than from the secretion. The urinary total testosterone also reflects androgen from other sources as well and might be expected to be less strongly correlated with serum testosterone levels.

As expected, the serum E_2 is very significantly correlated with the serum testosterone, because the serum testosterone is the source of most serum E_2 in normal men (Bardin, 1986). But also, both are highly bound to SHBG which would determine their serum concentrations.

V.3. 17β-Estradiol , atherogenic lipoprotein-lpids and HDL3

Over all, the data presented here suggest that the urinary excretion of unconjugated E_2 is directly associated with serum apoB, Lp(a) (for untransformed data only), and HDL_3 concentrations; while the urinary excretion of total E_2 is inversely correlated with cholesterol, LDL-C, apoB, and possibly HDL_3. Calculated serum unbound E_2 concentration and urinary unconjugated E_2 excretion had inverse and direct associations respectively with apoB levels after multiple linear regression which may be due to chance, as they were not significantly associated univariately. A similar explanation is proposed for the inverse correlation between HDL_3-C concentration and total urinary E_2 excretion. The most suggestive feature of this study is the finding of an inverse correlation between the urinary 17β-estradiol excretion rate and cholesterol, LDL-C and apoB which are strong risk factors for atherosclerosis (Levinson and Wagner, 1992).

Lp(a) concentration in men is rather stable throughtout life (Lawn, 1992). Apo(a) genes determine the total plasma Lp(a) concentrations, but, the apo(a) gene only accounts for approximately 40% of the variation in Lp(a) levels (Scanu & Fless, 1990), though a more recent paper shows that the apo(a) gene is estimated to be responsible for 91% of the variance of plasma Lp(a) concentration (Boerwinkle ***et al.,*** 1992). It has also been suggested that the LDL receptor gene may have an effect on plasma Lp(a) levels as in transgenic mice with overexpression of human low density lipoprotein receptors which accelerate the

the clearance of Lp(a), Lp(a) levels are low (Hofmann *et al.,* 1990). In subjects with familial hypercholesterolemia, who tend to elevated Lp(a) concentrations, there is defective LDL receptor function (Utermann, 1989). Therefore, factors affecting LDL receptor expression may have an effect on plasma Lp(a) concentrations. Estrogens are known to increase LDL-receptor activity (Seed, 1991). It is possible that estrogens in men affect LDL-receptor activity, reducing both apoB (LDL) and Lp(a) levels. It seems that the association between Lp(a) and LDL partly due to apoB may be through LDL receptor mediated by unconjugated E_2. As nicotinic acid is believed to have its effect on LDL levels by reducing VLDL synthesis it may lower Lp(a) (Gurakar *et al.,* 1985;, Carlson *et al.,* 1989; Schmidt *et al.,* 1993) by a similar mechanism. That may imply that lowering Lp(a) concentration occurs by reducing synthesis rather than by increasing its catabolism. The mechanism is thought to be by decreasing LDL production. There have been no suggestions to knowledge of estrogens reducing VLDL production. If these were shown to be in association with Lp(a), as for apoB, it might be exerting its effect on catabolism which might be regulated by serum unbound E_2. Recently fish oil has been noted to have the ability to lower Lp(a) level as well (Scanu and Fless, 1990; Fu *et al.,* 1991) although effects on LDL are not consistent or marked. On the other hand, HDL_3 level is also reduced by nicotinic acid (Blum *et al.,* 1977; Shepherd *et al.,* 1979) and fish oil (Mori *et al.,* 1991). It was suggested that the effect of nicotinic acid on the HDL subfraction distrubution may be mediated via the net transfer of apoA-I from HDL_3 to HDL_2 and/or via the reduction in apoA-II synthesis (Shepherd *et al.,* 1979).

In view of the effect of nicotinic acid and fish oil on Lp(a) and HDL_3 of which both have direct associations with urinary unconjugated E_2 excretion rate, (for Lp(a) at least parametrically, Pearson's $r=0.36618$ $p=0.0123$), it may be

that both HDL_3 and Lp(a) are affected by some common hormonal factors.

Moreover, in pathological states, such as diabetes, nephrotic syndromes and end-stage kidney disease, Lp(a) (Kapelrud, ***et al.,*** 1991; Jenkins ***et al.,*** 1991; Takegoshi ***et al.,*** 1990; Dieplinger ***et al.,*** 1993) and HDL_3 (Muls ***et al.,*** 1985; Short ***et al.,*** 1986) levels in serum are often found to be elevated. There has been a report of a decrease in serum apo(a) in patients after renal transplantation. This suggests that abnormal renal function affects Lp(a) metabolism (Black and Wilcken, 1992), although the mechanism is still unknown (Keane ***et al.,*** 1992).

V.4. Testosterone, and HDL-C and its subfractions

The association between total serum- and free-testosterone and total HDL-C reduces after adjustment for age, BMI, W/H ratio, SHBG and triglycerides, and the level of HDL is strongly inversely correlated with that of triglyceride in plasma (cf. **Table IV.5.A & Table IV.5.B**). An understanding of HDL-cholesterol is also complicated by the existence of the major subfractions HDL_2-C and HDL_3-C, and their interactions. Variation in total HDL is largely caused by changes in HDL_2 and the amount of HDL_3 in the blood of normal men is relatively constant (Anderson ***et al.,*** 1978; Wood and Haskell, 1979). Therefore it is expected that total HDL-C and HDL_2-C are strongly correlated univariately ($r=0.86426$, $p<0.0001$). It is interesting that HDL-C and HDL_3-C are also strongly correlated ($r=0.71815$, $p<0.0001$), and also as shown in this study (**Tables IV.5.A & IV.5.B**), the HDL_2-C subfraction has an association with testosterone, and HDL_3-C with estradiol. If HDL_2-C is the strongest predictor of both the presence and the extent of CAD as suggested in angiographic studies (Miller ***et al.,*** 1981, 1987; Drexel ***et al.,*** 1992) then an understanding of the association with testosterone levels may be important, because the association between total- and free- testosterone and HDL_2-C persists even after adjustment for age, BMI, WHR, SHBG, and triglyceride. HDL_2 is associated with apoA-I

and apoE which are involved in the metabolism of triglyceride-rich lipoprotein. This is demonstrated by the inverse correlation between HDL_2 and triglyceride in univariate correlation (Anderson, 1978; Patsch ***et al.,*** 1983, 1987). HDL_3-C was associated with apoA-I and apoA-II as shown both in humans (Blum ***et al.,*** 1977, Shepherd ***et al.,*** 1979) and in transgenic mice (Schultz ***et al.,*** 1992). To produce HDL_3 particles from HDL_2 it is necessary to remove about two-thirds of the cholesteryl ester molecules from the HDL_2 (Eisenberg, 1986) which is regulated by the enzyme, hepatic lipase (HL), and an appropriate amount of surface molecules from triglyceride-rich lipoproteins regulated by lipoprotein lipase (LPL) (Taskinen & Nikkila, 1981; Silverman ***et al.,*** 1993). After multiple linear regression (**Table IV.5.A & Table IV.5.B**), since HDL_3 is strongly positively correlated with the urinary unconjugated E_2 ($t=+2.8$, $p=0.0076$) and HDL_2 is positively correlated with serum total and unbound testosterone ($t=+2.60$, $p=0.0128$; $t=+2.94$, $p=0.0052$, respectively), the HL and/or LPL could possibly be under the control of unbound testosterone and/or E_2 in plasma. It should be noted that women have higher LPL activities than men. However, reduced LPL activity and enhanced HL activity leads to a reduction of total HDL due to decreased level of HDL_2 (Seed, 1991).

Chapter VI. Conclusions

Recently focus on the influence of the menopause on serum lipoprotein-lipids in healthy women has suggested that the association of menopause with potentially adverse changes in lipoprotein-lipids is independent of any effects of ageing but due either directly or indirectly to estrogen deficiency (Jensen ***et al.***, 1990; Stevenson ***et al.,*** 1993). Postmenopausal women have significantly higher concentrations of total cholesterol, LDL-C and HDL_3-C which are thought to be correlated with the reduction of estrogen production in the ovaries. In postmenopausal women, the reduction of estrogen levels, as compared to premenopausal women, has been shown to be associated with a pattern of lipoprotein-lipids profiles similar to that in men. After the menopause females and males have a similar risk for coronary heart disease. This study suggests that estradiol secretion may contribute to variation in lipoprotein-lipids in men. Since most of the estradiol in men originates from the transformation of testosterone, it is possible that there is a complex interaction between estradiol and testosterone as far as serum lipoprotein-lipids are concerned.

The unique feature of this study was the measurement of the urinary excretion rates of testosterone and estradiol. The measurement of 24-hour urinary excretion of sex hormones seemed likely to be more useful than that of plasma hormones because the urinary excretion rate of a hormone is expected to be a better index of its production or secretion rate, and the urinary excretion rate of unconjugated hormone might be a closer reflection of circulating biologically active hormone. In this study it is demonstrated that the secretion and production of estradiol, rather than that of testosterone, is inversely associated with the plasma concentrations of cholesterol, triglyceride, LDL-C, and apoB, and directly associated with those of HDL_3 and possibly Lp(a). It is unlikely these associations were due to the potential confounders BMI, SHBG, and triglyceride

because the association remained significant on stepwise multiple linear regression. The effect of E_2 on LDL-C concentrations is probably mediated through LDL-receptors. The effect on triglyceride concentrations may be mediated through E_2 effects on lipoprotein lipase, as estradiol increases the activities of LDL-receptors and lipoprotein lipase (Godsland ***et al.,*** 1987; Seed, 1991). The protection afforded to post-menopausal women on HRT (Knopp, 1988; Barrett-Connor and Bush, 1991) may also be partly mediated by antithrombotic effects on the arterial wall (Adams ***et al.,*** 1990). However, other reports have suggested that men with CAD actually have higher estrogen levels than men without disease (Phillips[1,2], 1993) implying that any beneficial effect on lipoproteins in men from higher estrogen levels provides no protection. It must be admitted, at this point, that the existence of correlations is not proof of metabolic cause and effect. Further studies in this area should be pursued for these might be helpful in the elucidation of the relation between sex hormone secretion in normal and abnormal lipid states. Lp(a) and HDL_3-C levels are both high in nephropathy (Keane ***et al.***, 1990, 1992). Perhaps disturbance in sex-hormone levels should be considered as possible contributions.

HDL-cholesterol, including its subfraction HDL_2-C is associated with testosterone secretion and production. In this regard, the present results in 46 Chinese male subjects show that the ***serum testosterone*** persistently has a ***direct*** correlation with HDL-C and/or HDL_2-C, which is in agreement with Heller ***et al.*** (1983), Lichtenstein ***et al.*** (1987), and Barrett-Conner and Khaw (1988), but not with the previous studies of Semmens ***et al.***(1983), Goldberg ***et al.*** (1985), and Stefanick ***et al.*** (1987). As HDL_2 is metabolically linked with triglyceride-rich lipoproteins (Patsch ***et al.***, 1983, 1987, 1992; Silverman ***et al.,*** 1993) the association between testosterone in men and postprandial lipemia (or the clearance of triglyceride-rich lipoproteins) possibly is a topic worthwhile for

future studies.

In summary, the above findings suggest that sex hormones have effects on lipoprotein-lipids concentrations in serum and may therefore play a role in the development of coronary disease.

References

Adams, M. R., J. R. Kaplan, S. B. Maunck, D. R. Koritnik, J. S. Parks, M. S. Wolfe, and T. B. Clarkson. (1990) Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys. Lack of an effect of added progesterone. Arteriosclerosis, **10:** 1051-1057

Amatruda, J. M., S. M. Harman, G. Pourmotabbed, and D. H. Lockwood. (1978) Depressed plasma testosterone and fractional binding of testosterone in obese males. J. Clin. Endocrinol. Metab. **47:** 268-271

Anderson, D. C. (1974) Sex hormone-binding globulin. Clin. Endocrinol. (Oxf) 3:69-96

Anderson, D. W. (1978) H.D.L. cholesterol: The variable components. **Lancet:** 819-820

Anderson, D. W., A. V. Nichols, S. S. Pan, and F. T. Lindgren. (1978) High density lipoprotein distribution: resolution and determination of three major components in a normal population sample. Atherosclerosis **29:** 161-179

Atger, V., E. Wirbel, D. Roche, M. Apfelbaum, M. Burstein, and A. Girard-Globa. (1990) Distribution of HDL_2 and HDL_3 in a random population of healthy French males and females--evalution by a two-step precipitation procedure. Clin Chim. Acta.**189:** 111-122

Austin, M. A. (1989) Plasma triglyceride as a risk factor for coronary heart disease: the epidemiologic evidence and beyond. Am. J. Epidemiol. **129:** 249-259

Bachorik, P. S., and J. J. Albers. (1986) Precipitation methods for quantification of lipoproteins. In "Methods in Enzymology" Vol. 129 (ed. Albers, J.J., Segrest, J.P.), Academic Press, London. pp. 78-100

Badimon, J. J., V. Fuster, and L. Badimon. (1992) Role of high density lipoproteins in the regression of atherosclerosis. Circulation, **86 (Suppl. III):** III86-III94

Barbir, M.., D. Wile, I. Trayver, V. R. Aber, and G. R. Thompson. (1988) High prevalence of hypertriglyceridaemia and apolipoprotein abnormalities in coronary artery disease. Br. Heart J. 60:397-403

Bardin, C.W. (1986) Pituitary-testicular axis. In "Reproductive Endocrinology" ed. by Yen, S.S.C. and Jaffe, R.B. 2nd ed., 1986, W.B. Saunders Co., Philadelphia.

Barrett-Connor, E., K-T. Khaw. (1988) Endogenous sex hormones and cardiovascular disease in men. A prospective population-based study. Circulation, **78**: 539-545

Barrett-Connor, E. and T. L Bush. (1991) Estrogen and coronary heart disease

in women. JAMA. **265:** 1861-1867

Bhatnagar, D., and P. N. Durrington. (1991) Clinical value of apolipoprotein measurement. Ann. Clin. Biochem. **28:** 427-437

Black, I. W. and D. E. L. Wilcken. (1992) Decreases in apolipoprotein(a) after renal transplantation: Implication for lipoprotein(a) metabolism. Clin. Chem. **38 (3):** 353-357

Blackman, M. R. (1989) Aging. In "Endocrinology" vol. 3, 2nd ed., 1989, Degroot, L. J.(editor), W. B. Saunders Co., Philadelphia.

Blum, C. B., R. I. Levy, S. Eisenberg, M. Hall, III, R. H. Goebel, and M. Berman. (1977) High density lipoprotein metabolism in man. J. Clin. Invest. **60:** 795-807

Boerwinkle, E., C. C. Leffert, J. Lin, C. Lackner, G. Chiesa, and H. H. Hobbs. (1992) Apolipoprotein (a) gene accounts for greater than 90% of the variation in plasma lipoprotein (a) concentrations. J. Clin. Invest. **90:** 52-60

Bogicevic, M., and V. Stefanovic. (1988) Relationship between parathyroid hormone and pituitary-testicular axis in patients on maintenance hemodialysis. Exp. Clin. Endocrinol.**92(3):** 357-362

Borkman, M., L. H. Storlien, D. A. Pan, A. B. Jenkins, D. J. Chisholm, and L. V. Campbell. (1993) The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. N. Engl. J. Med. **328:** 238-244

Bray, G. A. (1989) Obesity: An endocrine perspective. In "Endocrinology" Vol.3, 2nd ed., L. J. DeGroot (editor), 1989, W.B. Saunders Co.

Bush, T. L., E. Barrett-Connor, L. D. Cowan, M. H. Criqui, R. B. Wallace, C. M. Suchindran, H. A. Tyroler, and B. M. Rifkind. (1987) Cardiovascular mortality and noncontraceptive use of estrogen in women: Results from the Lipid Research Clinics Program Follow-Up Study. Circulation, **75:** 1102-1109

Brown, M. S. and J. L. Goldstein. (1986) A receptor-mediated pathway for cholesterol homeostasis. Science, **232:** 34-47

Camacho, A.M. and C. J. Migeon. (1964) Studies on the origin of testosterone in the urine of normal adult subjects and patients with various endocrine disorders. J. Clin. Invest. **43:** 1083-1089

Carlson, L. A., A. Hamsten and A. Asplund. (1989) Pronounced lowering of serum levels of lipoprotein(a) in hyperlipidaemic subjects treated with nicotinic acid. J. Int. Med. **226:** 271-276

Castelli, W. P., T. Gordon, M. C. Hjortland, A. Kagan, J. T. Doyle, C. G. Hames, S. B. Hulley, and W. J. Zukel. (1977) Alcohol and blood lipids -- the cooperative lipoprotein phenotype study. **Lancet:**153-155

Chambless, L. E., R. P. McMahon, S. A. Brown, W Patsch, G. Heiss, and Y-

L. Shen. (1992) Short-term intraindividual variability in lipoprotein measurements: The atherosclerosis risk in communities (ARIC) study. Am.J. Epidemiol. 136(9):1069-1081

Chard, T. (1990) An Introduction to Radioimmunoassay and Related Techniques. 4th revised ed. In "Laboratory Techniques in Biochemistry and Molecular Biology" Volume 6, Part II. Burdon, R. H. and van Knippenberg, P. H. (eds), Elsevier, Amsterdam, New York, Oxford.

Cheung, M. C., A. J. Mendez, A.C.Wolf, and R. H. Knopp. (1993) Characterization of apolipoprotein A-I- and A-II-containing lipoproteins in a new case of high density lipoprotein deficiency resembling Tangier disease and their effects on intracellular cholesterol efflux. J. Clin. Invest. **91**: 522-529.

Christeff, N., C. Benassayag, C. Carli-Vielle, A. Carli, and E. A. Nunez. (1988) Elevated estrogen and reduced testosterone levels in the serum of male septic shock patients. J. Steroid Biochem. **29(4)**: 435-440

Contreras, P., E. Altieri, C. Liberman, A. Gac, A. Rojas, A. Ibarra, M. Ravanal, and M. Serón-ferré. (1985) Adrenal rest tumor of the liver causing cushing's syndrome: Treatment with ketoconazole preceding an apparent surgical cure. J. Clin. Endocrinol. Metab. **60(1)**: 21-28

Criqui, M. H., G. Heiss, R. Cohn, L. D. Cowan, C. M. Suchindran, S. Bangdiwala, S. Kritchevsky, D. R. Jacobs Jr., H. K. O'Grady, and C. E. Davis. (1993) Plasma triglyceride level snd mortality from coronary heart disease. N. Engl. J. Med. **328:** 1220-1225

Dahlen, G. H., J. R. Guyton, M. Attar, J. A. Farmer, J. A. Kautz, and A. M. Gotto, Jr. (1986) Association of levels of lipoprotein Lp(a), plasma lipids, and other lipoproteins with coronary artery disease documented by angiography. Circulation, **74(4):** 758-765

Dai, W. S., L. H. Kuller, R. E. LaPorte, J. P. Gutai, L. Falvo-Gerard and A. Caggiula. (1981) The epidemiology of plasma testosterone levels in middle-aged men. Am. J. Epidemiology, **114:** 804-816

Derr, J., P. M. Kris-Etherton, T. A. Pearson, and F. H. Seligson (1993) The role of fatty acid saturation on plasma lipids, lipoproteins, and apolipoproteins: II. The plasma total and low-density lipoprotein cholesterol response of individual fatty acid. Metabolism, **42 (1):** 130-134

Deschenes, M. R.,W. J. Kraemer, C. M. Maresh, and J. F. Crivello. (1991) Exercise-induced hormonal changes and their effects upon skeletal muscle tissue. Sports Medicine **12(2):** 80-93

Devenport, M. H., D. Crook, V. Wynn, L. J. Lees. (1989) Metabolic effects of low-dose fluconazole in healthy female users and non-users of oral contraceptives. Br. J. Clin. Pharmacol. **27(6):** 851-859

Dieplinger, H., C. Lackner, F. Kronenberg, C. Sandhozer, K. Lhotta, F.

Hoppichler, H. Graf, and P. König. (1993) Elevated plasma concentrations of lipoprotein (a) in patients with end-stage renal disease are not related to the size polymorphism of apolipoprotein (a). J. Clin. Invest. **91:** 397-401

Doerr, P. and K. M. Pirke. (1976) Cortisol-induced suppression of plasma testosterone in normal adult males. J. Clin. Endocrinol. Metab. **43:** 622-629

Drexel, H., F. W. Amann, K. Rentsch, C. Neuenschwander, A. Luethy, S. I. Khan, and F. Follath. (1992) Relation of the level of high-density lipoprotein subfractions to the presence and extent of coronary artery disease. Am. J. Cardiology, **70:** 436-440

Edén, S. O. Wiklund, J. Oscarsson, T. Rosén, and B-Å. Bemgtsspm. (1993) Growth hormone treatment of growth hormone-deficient adults results in a marked increase in Lp(a) and HDL cholesterol concentrations. Arteriosclerosis and Thrombosis, **13:** 296-301

Eisenberg, S. (1986) Plasma lipoprotein conversion. In "Methods in Enzymology" vol. 129, pt.B. Albers, J. J. and J. P. Segrest (eds.), 1986, Academic Press, Inc., Orlando, San Diago, N.Y.

Falk, O., E. Palonek, and I. Bjorkhem. (1988) Effect of ethanol on the ratio between testosterone and epitestosterone. Clin. Chem. **34(7):**1462-1464

Farish, E., C. D. Fletcher, D. M. Hart, and M. L. Smith. (1990) Effects of Bilateral oophorectomy on lipoprotein metabolism. Br. J. Obstet. Gynaecol. **97**: 78-82

Farish, E., H. A. Rolton, J. F. Barnes, and D. M. Hart. (1991) Lipoprotein (a) concentrations in postmenopausal women taking norethisterone. BMJ. **303:** 694

Fenske, M. (1988) Urinary excretion of free glucocorticosteroids and testosterone in the mongolian gerbil (Meriones unguiculatus): Effects of long-acting corticotrophin and humangonadotrophin. Comp. Biochem. Physiol. **91(A):** 789-795

Fenske, M. (1989) Urinary excretion of free progestins, androgens, and estradiol after injection of (1-24) ACTH in the mongolian gerbil. Life Science, **45:** 1177-1184

Feussner, G., A-M. Wingen, and R. Ziegler. (1990) Type III hyperlipoproteinemia in a child with hemolytic uremic syndrome. Metabolism, **39 (11):** 1196-1199

Fielding C. T. and P. E. Fielding. (1981) Evidence for a lipoprotein carrier in human plasma catalyzing sterol efflux from cultured fibroblasts and its relationship to lecithin: cholesterol acyltransferase. Proc. Natl. Acad. Sci. **78 (6):** 3911-3914

Forbes, G. B., M. R. Brown, S. L. Welle, and L. E. Underwood. (1989)

Hormonal response to overfeeding. Am. J. Clin. Nutri. **49(4):**608-611

Fraser, C. G. (1986) Interpretation of Clinical Chemistry Laboratory Data. Blackwell Scientific Publications. Oxford, Edinburgh, London. Chapter six, pp.111-135

Fraser, C. G., and E. K. Harris. (1989) Generation and application of data on biological variation in clinical chemistry. Critical Reviews in Clin. Lab. Sci. **27 (5):** 409-437

Friedl, K. E., C. J. Hannan, Jr, R. E. Jones, and S. R. Plymate. (1990) High-density lipoprotein cholesterol is not decreased if an aromatizable androgen is administered. Metabolism, **39 (1)**: 69-74

Friedman, M. and Rosenman, R. H. (1975) Type A behaviour and your heart. Fawcett Crest Book, Greenwich, Connecticut, USA.

Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. (1972) Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of preparative ultracentrifuge. Clin. Chem. **18:**499

Fu, B., W. Terres, M. Orgass, H. Greten. (1991) Dietary fish oil lowers lipoprotein(a) in primary hypertriglyceridemia. (Abstract only). Atherosclerosis (Ireland), **90 (1):** 95-97

Funke, H., A. v. Eckardstein, P. H. Pritchard, M. Karas, J. J. Albers, and G. Assmann. (1991) A Frameshift mutation in the human apolipoprotein A-I gene causes high density lipoprotein deficiency, partial lecithin: cholesterol-acytransferase deficiency, and corneal opacities. J. Clin. Invest. **87**: 371-376

Fuster, V., J. J. Badimon and L. Badimon. (1992) Clinical-pathological correlation of coronary disease progression and regression. Circulation, **86(Suppl. III):** III-1-III-11

Galbo, H. (1991) General principles for biochemical responses to exercise. Scand. J. Clin. Lab. Invest. **51 (Suppl. 211):**32-33

Gerlo E. A. M., D. F. Schoors, A. G. and Dupont (1991) Age- and Sex- related differences for the urinary excretion of Norepinephrine, Epinephrine, and Dopamine in adults. Clin. Chem. **37(6):** 875-878

Godsland, I. F., V. Wynn, D. Crook, and N. E. Miller. (1987) Sex, plasma lipoproteins, and atherosclerosis. Am Heart J. **114:** 1467-1503

Goldberg, R. B., D. Rabin, A. N. Alexander, G. C. Doelle, and G. S. Getz. (1985) Suppression of plasma testosterone leads to an increase in serum total and high density lipoprotein cholesterol and apoproteins A-I and B. J. Clin. Endocrinol. Metab. **60**: 203-207

Gordon, D. J., J. L. Probstfield, R. J. Garrison, J. D. Neaton, W. P. Castelli, J. D. Knoke, D. R. Jacobs Jr., S. Bangdiwala, and H. A. Tyroler. (1989) High-density lipoprotein cholesterol and cardiovascular disease ---Four

prospective American studies. Circulation, **79:** 8-15

Gurakar, A. J. M. Hoeg, G. Kostner, N. M. Papadopoulos and H. B. Brewer, Jr. (1985) Levels of lipoprotein Lp(a) decline with neo-nycin and niacin treatment. Atherosclerosis **57:** 293-301

Hackney, A., Sinning, W., and Bruot, B. (1990) Hypothalamic-pituitary-testicular axis function in endurance-trained males. Intern. J. Sports Medicine, **11:** 298-303

Haning, R., G. P. Orczyk, B. V. Caldwell, and H. R. Behrman. (1979) Plasma estradiol, estrone, estriol and urinary estriol glucuronide. In "Methods of Hormone Radioimmunoassay", 2nd ed., pp. 675-700. Jaffe, B. M. and H. R. Behrman (eds.). Academic Press, New York, San Francisco, London.

Heiss, G., I. Tamir, C. E. Davis, H. A. Tyroler, B. M. Rifkind, G. Schonfeld, D. Jacobs, and I. D. Frantz. (1980) Lipoprotein cholesterol distributions in selected North American populations: the Lipid Research Clinics Program Prevalence Study. Circulation, **61:** 302-315

Heller, R. F, M. J. Wheeler, J. Micallef, N. E. Miller, and B. Lewis. (1983) Relationship of high density lipoprotein cholesterol with total and free testosterone and sex hormone binding globulin. Acta Endocrinologica 104: 253-256

Henriksson, P., B. Angelin, and L. Berglund. (1992) Hormonal regulation of serum Lp(a) levels. J. Clin. Invest. **89:** 1166-1171

Hill, P., E. Wynder, L. Garbaczewski, H. Garnes, A.R.P.Walker, and P. Helman. (1980) Plasma hormones and lipids in men at different risk for coronary heart disease. Am. J. Clin. Nutr. **33:** 1010-1018

Hofmann, S. L., D. L. Eaton, M. S. Brown, W. J. McConathy, J. L. Goldstein, and R. E. Hammer. (1990) Overexpression of human low density lipoprotein receptors leads to accelerated catabolism of Lp(a) lipoprotein in transgenic mice. J. Clin. Invest. **85:** 1542-1547

Horton, R. (1989) Testicular steroid secretions, metabolism, and mode of action. In "Endocrinology" vol.3, 2nd ed., 1989 DeGroot, L.J. (editor), W. B. Saunders Co., Philadelphia.

Houlston, R. and W. Friedl. (1988) Biochemistry and clinical significance of lipoprotein (a). Ann. Clin. Biochem. **25:** 499-503

Houmard, J. A., W. S. Wheeler, M. R. McCammon, and D. Holbert, (1991) Effects of fitness level and the regional distribution of fat on carbohydrate metabolism and plasma lipids in middle to older-aged man. Metabolism **40(7):** 714-719

Hromadová, M., T. Hacik, and I. Riecansky. (1985) Concentration of lipid, apoprotein B and testosterone in patients with coronarographic findings. Klin-Wochenschr. **63(20):** 1071-1074 (abstract).

Hromadová, M., T. Hácik, E. Malatinský, A. Sklovský, J. Cervenakov, and F. Lábady. (1991) Lipid metabolism in young males with hypotestosteronaemia and oligospermia prior to, during, and after treatment. Intern. Urol. Nephr. **23 (1):** 69-75

Hughes, G. S., T. V. Ringer, K. C. Watts, M. J. DeLoof, S. F. Francom, and C. R. Spillers. (1990) Fish oil produces an atherogenic lipid profile in hypertensive men. Atherosclerosis, **84(2-3):** 229-237

James, R. W., A. Proudfoot and D. Pometta. (1989) Immunoaffinity fractionation of high-density lipoprotein subclasses 2 and 3 using anti-apolipoprotein A-I and A-II immunosorbent gels. Biochim. Biophys. Acta, **1002:** 292-301

James, R. W. and D. Pometta. (1990) Immunofractionation of high density lipoprotein subclasses 2 and 3. Similarities and differences of fractions isolated from male and female populations. Artherosclerosis, **83:** 35-45

Jenkins, A. J., J. S. Steele, E. D. Janus, J. D. Santamaria and J. D. Best. (1991) Increased plasma apo(a) levels in diabetic patients with macrovascular disease, microalbuminuria and albuminuria. Clin. Biochem. Revs. **12:** 69

Jensen, J., L. Nilas and C. Christiansen. (1990) Influence of menopause on serum lipids and lipoproteins. Maturitas, **12:** 321-331

Johansson, J., L. A. Carlson, C. Landou, and A. Hamsten. (1991) High density lipoproteins and coronary atherosclerosis--A strong inverse relation with the largest particles is confined to normotriglyceridemic patients. Arteriosclerosis and Thrombosis, **11**:174-182

Kannel, W. B. and P. W. F. Wilson. (1992) Efficacy of lipid profiles in prediction of coronary disease. Am. Heart J. **124 (3):** 768-774

Kapelrud, H., H-J. Bangstad, K. Dahl-Jørgensen, K. Berg, and K. F. Hanssen. (1991) Serum Lp(a) lipoprotein concentrations in insulin dependent diabetic patients with microalbuminuria. BMJ, **303:** 675-678

Keane, W. F. and B. L. Kasiske. (1990) Hyperlipidemia in the nephrotic syndrome. N. Eng. J. Med. **323:** 604-606

Keane, W. F. , J. V. St. Peter, and B. L. Kasiske. (1992) Is the aggressive management of hyperlipidemia in nephrotic syndrome mandatory? Kidney Int. **42** (Suppl..38): S134-S141

Kesäniemi, Y. A., C. Ehnholm, and T. A. Miettinen (1987) Intestinal cholesterol absorption efficiency in man is related to apoprotein E phenotype. J. Clin. Invest. **80:** 578-581

Khaw, K-T. and E. Barrett-Connor. (1991) Endogenous sex hormones, high density lipoprotein cholesterol, and other lipoprotein fractions in men. Arteriosclerosis and Thrombosis **11**: 489-494

Kiel, D. P., J. A. Baron, S. R. Plymate, and C. G. Chute. (1989) Sex Hormones and lipoproteins in men. The Am. J. Med. **87:** 35- 39.

Kiens, B. (1992) Lipoprotein metabolism in exercise. Scand. J. Clin. Lab. Invest. 52 (suppl. 211): 36

Knopp, R. H. (1988) The effects of postmenopausal estrogen therapy on the incidence of arteriosclerotic vascular disease. Obstet. Gynecol. **72**: 23S-30S

Korenman, S. G. and M. B. Lipsett. (1964) Is testosterone glucuronoside uniquely derived from plasma testosterone? J. Clin. Invest. **43(11):** 2125-2131

Kouchiyama, S., T. Shinozaki, S. Masuyama, K Tatsumi, H.. Kimura, and T. Kuriyama. (1989) [Depression of testosterone secretion in male patients with respiratory failure.] Nippon-Kyobu-Shikkan-Gakkai-Zasshi, **27(3):** 345-351 (abstract)

Krempler, P., G. M. Kostner, K. Bolzano, and F. Sandhofer. (1980) Turnover of lipoprotein(a) in man. J. Clin. Invest. **65:** 1483-1490

Kuller, L. H., J. P. Gutai, E. Meilahn, K. A. Matthews, and P. Plantinga. (1990) Relationship of endogenous sex steroid hormones to lipids and apoproteins in postmenopausal women. Arteriosclerosis, **10:** 1058-1066

Lawn, R. M. (1992) Lipoprotein(a) in heart disease. Scientific American, **June:** 26-32

Levinson, S. S. and S. G. Wagner. (1992) Measurement of apolipoprotein B-containing lipoproteins for routine clinical laboratory use in cardiovascular disease. Arch. Pathol. Lab. Med. **116**: 1350-1354

Lichtenstein, M.J., J. W. Yarnell, P. C. Elwood, A. D. Beswick, P. M. Sweetnam, V. Marks, D. Teale, and D. Riad-Fahmy. (1987) Sex hormones, insulin, lipids and prevalent ischemic heart disease. Am. J. Epidemiol. **126(4):** 647-657

Lipsett, M. B., C. J. Migeon, M. A. Kirschner, and C. W. Bardin. (1968) Physiologic basis of disorders of androgen metabolism. Ann. Intern. Med. **68(6):** 1327-1344

Lipsett, M.B. (1986) Steroid hormones. In "Reproductive Endocrinology" 2nd ed. 1986. Yen, S.S.C. and Jaffe, R.B. (eds.), W. B. Saunders Co., Philadelphia.

Mahley, R. W., and T. L. Innerarity. (1983) Lipoprotein receptors and cholesterol homeostasis. Biochim. Biophys. Acta. **737**: 197-222.

Mahley, R. W. (1988) Apoprotein E: Cholesterol transport protein with expanding role in cell biology. Science, **240:** 623-630

Marinetti, G.V. (1990) Disorders of Lipid Metabolism, Plenum Press, New York and London.

Martini, S., G. Baggio, L. Baroni, G. Baldo Enzi, R. Fellin, M. R. Baiocchi and G. Crepaldi. (1984) Evaluation of HDL_2 and HDL_3 cholesterol by a precipitation procedure in a normal population and in different

hyperlipidemic phenotypes. Clin. Chim. Acta. **137:** 291-298

Masarei, J.R.L., Armstrong, B.K., Skinner, A.W., Ratajczak, T., Hahnel, R., Crooke, D., and Clarke, H.T. (1980) HDL- cholesterol and sex-hormone status. Lancet, 208

Masarei, J. R. L., I. L. Rouse, .W. J. Lynch, K. Robertson, R. Vandongen, and L. J. Beilin. (1984) Vegetarian diets, lipid and cardiovascular risk. Aust-N-Z-Med. **14 (4):** 400-404

Masarei, J. R. L., I. B. Puddey, I. L. Rouse, W. J. Lynch, R. Vandongen, and L. J. Beilin. (1986) Effects of alcohol consumption on serum lipoprotein-lipid and apolipoprotein concentrations. Results from an intervention study in healthy subjects. Atherosclerosis, **60 (1)**: 79-87

Masarei, J. R. L, I. B. Puddey, R. Vandongen, L. J. Beilin and W. J. Lynch. (1991) Effect of smoking cessation on serum apolipoprotein A-I and A-II concentration. Pathology, **23:** 98-102

Mattews, K. A., E. Meilahn, L. H. Kuller, S. F. Kelsey, A. W. Caggiula, and R. R. Wing. (1989) Menopause and risk factors for coronary heart disease. N. Engl. J. Med. **321**: 641-646

Mendoza, S. G., H. Carrasco, A. Zerpa, Y. Briceno, F. Rodriguez, J. Speirs, C. J. Glueck. (1991) Effect of physical training on lipids, lipoproteins, apolipoproteins, lipases, and endogenous sex hormones in men with premature myocardial infarction. Metabolism **40(4):** 368-377

Micheli, A., P. Muti, P. Pisani, G. Secreto, C. Recchione, A. Totis, R. Fissi, A. Cavalleri, S. Panico, and F. Berrino. (1991) Repeated serum and urinary androgen measurements in premenopausal and postmenopausal women. J. Clin. Epidemiol., **44(10):**1055-1061

Miller, N. E., F. Hammett, S. Saltissi, S. Rao, H. van Zeller, J. Coltart, and B. Lewis. (1981) Relation of angiographically defined coronary artery disease to plasma lipoprotein subfractions and apolipoproteins. B. M. J. **282**:1741-1744

Miller, N. E. (1987) Association of high-density lipoprotein subclasses and apolipoproteins with ischemic heart disease and coronary atherosclerosis. Am. Heart J. 113 **(2):** 589-597

Mishra, L., N-A. Le, W. V. Brown, and E. Mezey. (1991) Effect of acute intravenous alcohol on plasma lipoproteins in man. Metabolism **40 (11):** 1128-1130

Mori, T. A., R. Vandongen, J. R. L. Masarei, I. L. Rouse, and D. Dunbar (1991) Comparison of diets supplemented with fish oil or olive oil on plasma lipoproteins in insulin-dependent diabetics. Metabolism, **40(3):** 241-246

Muls, E. M. Rosseneu, R. Daneels, M. Schurgess, and J. Boelaert.(1985) Lipoprotein distribution and composition in the human nephrotic

syndrome. Atherosclerosis **54:** 225-237

Myant, N.B. (1991) "Cholesterol" in "Encyclopedia of Human Biology" vol.2, pp. 411-418. R. Dulbecco, editor-in-chief, Academic Press, Inc., San Diego, New York, Boston. 1991.

Nahoul, K. and M. Roger. (1990) Age-related decline of plasma bioavailable testosterone in adult men. J. Steroid Biochem. **35 (2):** 293-299

Nanjee, M.N. and Miller, N.E. (1989) Plasma lipoproteins and adrenocortical hormones in men--positive association of low density lipoprotein cholesterol with plasma cortisol concentration. Clin. Chim. Acta 180:113-120

Nestler, J. E. (1993) Editorial: Sex hormone-binding globulin: a marker for hyperinsulinemia and/or insulin resistance? J. Clin. Endocrinol. Metab. **76 (2):** 273-274

Orentreich, N., J. L.Brind, R. L. Rizer, and J. H. Vogelman. (1984) Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations through adult- hood. J. Clin. Endocrinol. Metab. **59:**551

Panteghini, M. and F. Pagani. (1991) Serum concentration of lipoprotein(a) during normal pregnancy and postpartum. Clin. Chem. **37 (11):** 2009-2010

Pardridge, W.M. (1991) Bioavailable testosterone in salivary glands. Clin. Chem. **37(2):**139-140

Pasquali, R., F. Casimirri, N. Melchionda, R. Fabbri, M. Capelli, L. Platè, D. Patrono, V. Balestra, and L. Barbgara. (1988) Weight loss and sex steroid metabolism in massively obese man. J. Endocrinol. Invest. **11(3):**205-210

Pasquali, R., F. Casimirri, S. Cantobelli, N. Melchionda, A. M Morselli Labate, R. Fabbri, M. Capelli, and L. Bortoluzzi (1991) Effect of obesity and body fat distribution on sex hormones and insulin in men. Metabolism, **40 (1):** 101-104

Patsch, J. R., J. B. Karlin, L. W. Scott, L. C. Smith, and A. M Gotto, Jr. (1983) Inverse relationship between blood levels of high density lipoprotein subfraction 2 and magnitude of postprandial lipema. Proc. Natl. Acad. Sci. **80:** 1449-1453

Patsch, J. R., S. Prasad, A. M. Gotto, Jr., and W. Patsch. (1987) Relationship of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase. J. Clin. Invest. **80:** 341-347

Patsch, J. R., G. Miesenböck, T. Hopferwieser, V. Mühlberger, E. Knapp, J. Kay Dunn, A. M. Gotto Jr., and W. Patsch. (1992) Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. Arteriosclerosis and Thrombosis, **12:** 1336-1345

Peiris, A. N., J. I., Stagner, S. R. Plymate, R. L. Vogel, M. Heck and E. Samols. (1993) Relationship of insulin secretory pulses to sex hormone-binding

globulin in normal men. J. Clin. Endocrinol. Metab. **76 (2):** 279-282

Phillips[1], G. B. (1993) Relationship between serum sex hormones and the glucose-insulin-lipid defect in men with obesity. Metabolism, **42 (1):** 116-120

Phillips[2], G. B. (1993) Relationship of serum sex hormones to coronary heart disease. Steroids, **58:** 286-290

Preziosi, P., E. Barrett-Connor, L. Papoz, M. Roger, M. Saint-Paul, K. Nahoul, and D. Simon. (1993) Interrelation between plasma sex hormone-binding globulin and plasma insulin in healthy adult women: the Telecom Study. J. Clin. Endocrinol. Metab. **76 (2):** 283-287

Puchois, p., A. Kandoussi, P. Fievet, J. L. Fourrier, M. Bertrand, E. Koren and J. C. Fruchart. (1987) Apolipoprotein A-I containing lipoproteins in coronary artery disease. Atherosclerosis, **68:** 35-40

Puddey, I. B., J. R. Masarei, R. Vandongen, and L. J. Beilin (1986) Serum apolipoprotein A-II as a marker of change in alcohol intake in male drinkers. Alcohol-Alcohol, **21(4):** 375-383 (Abstract).

Quivers, E. S., D. J. Driscoll, C. D. Garvey, A. M. Harris, J. Harrison, D. M. Huse, P. Murtaugh, and W. H. Weidman (1992) Variability in response to a low-fat, low-cholesterol diet in children with elevated low-density lipoprotein cholesterol levels. Pediatrics, **89:** 925-929

Reed, M. J., R. W. Cheng, M. Simmonds, W. Richmond, and James, V.H.T.(1987) Dietary lipids: an additional regulation of plasma levels of sex hormone-binding globulin. J. Clin. Endocrinol. Metab. **64(5):**1083-1085

Sacks, F.M., Castelli, W.P., Donner, A., and Kass, E.H. (1975) Plasma lipids and lipoproteins in vegetarians and controls. The New England Journal of Medicine, 292(22):1148-1151

Sandholzer[1], C., E. Boerwinkle, N. Saha, M. C. Tong, and G. Utermann. (1992) Apolipoprotein (a) phenotypes, Lp(a) concentration and plasma lipid levels in relation to coronary heart disease in a Chinese population: Evidence for the role of the apo(a) gene in coronary heart disease. J. Clin. Invest. **89:** 1040-1046

Sandholzer[2], C., N. Saha, J. D. Kark, A. Rees, W. Jaross, H. Dieplinger, F. Hoppichler, E. Boerwinkle, and G. Utermann. (1992) Apo(a) isoforms predict risk for coronary heart disease. A study in six populations. Arteriosclerosis and Thrombosis, **12:** 1214-1226

Scanu, A. M. and G. M. Fless. (1990) Lipoprotein(a)-heterogeneity and biological relevance. J. Clin. Invest. **85:** 1709-1715

Schmidt, E. B., D. R. Illingworth, S. Bacon, S. J. Russell, S. R. Thatcher, R. W. Mahley, and K. H. Weisgraber. (1993) Hypolipidemic effects of nicotinic acid in patients with familial defective apolipoprotein B-100.

Metabolism, **42 (2):** 137-139

Schultz, J. R., E. L. Gong, M. R. McCall, A. V. Nichols, S. M. Clift, and E. M. Rubin. (1992) Expression of human apolipoprotein A-II and its effect on high density lipoproteins in transgenic mice. J. Biol. Chem. **267**(30):21630-21636

Scott, M. G. (1993) Primary prevention of coronary heart disease with drug therapy. Safty and total mortality issues. JAMA (SEA), **January**: 36-37, 1993

Seed, M. (1991) Sex hormones, lipoproteins, and cardiovascular risk. Atherosclerosis, **90**: 1-7

Segal, K.R., A. Dunaif, B. Gutin, J. Albu, A. Nyman, and F.x. Pi-Sunyer. (1987) Body composition, not body weight, is related to cardiovascular disease risk factors and sex hormone levels in men. J. Clin. Invest. **80(4):**1050-1055

Seidell, J.C., M. Cigolini, P. Deurenberg, A. Oosterlee, and G. Doornbos. (1989) Fat distribution, androgens, and metabolism in non-obese women. Am. J. Clin. Nutr. **50(2):** 269- 273

Semmens, J., I. Rouse, L. J. Belin, and J. R. L. Masarei. (1983) Relationship of plasma HDL-cholesterol to testosterone, estradiol, and sex-hormone-globulin levels in men and women. Metabolism **32 (5):** 428-432

Sewdarsen, M., S. Vythilingum, I. Jialal, R.K. Desai, P. Becker. (1990) Abnormalities in sex hormones are a risk factor for premature manifestation of coronary artery disease in south african Indian men. Atherosclerosis, **83(2-3):**111-117

Shepherd, J., C. J. Packard, J. R. Patsch, A. M. Gotto and O. D. Taunton. (1979) Effects of nicotinic acid therapy on plasma high density lipoprotein subfraction distribution and composition and on apolipoprotein. J. Clin. Invest. **63:** 858-867

Short, C. D., P. N. Durrington, N. P. Mallick, L. P. Hunt, L. Tetlow, and M. Ishola. (1986) Serum and urinary high density lipoproteins in glomerular disease with proteinuria. Kidney Int. **29:** 1224-1228

Silverman, D. I., G. S. Ginsburg, and R. C. Pasternak. (1993) High-density lipoprotein subfractions. Am. J. Med. **94:** 636-645

Slowinska-Srzednicka, J., S. Zgliczynski, M. Ciswicka-Snzajderman, M. Srzednicki, P. Soszynski, M. Biernacka, M. Woroszylska, W. Ruzyllo, and Z. Sadowski. (1989) Decreased plasma dehydro- epiandrosterone sulfate and di- hydrotestosterone concentrations in young men after myocardial infarction. Atherosclerosis, **79(2-3):**197-203

Smith, K. D. and L. J. Rodriguez-Rigau. (1989) Laboratory evaluation of testicular function. In "Endocrinology" vol.3, 2nd ed., 1989, DeGroot,

L.J. (editor), W. B. Saunders Co., Philadelphia.

Södergård, R., T. Bäckström, V. Shanbhag, and H. Carstènsen. (1982) Calculation of free and bound fractions of testosterone and estradiol-17β to human plasma proteins at body temperature. J. Steroid Biochem. **16:** 801-810

Stefanick, M. L., P. T. Williams, R. M. Krauss, R. B. Terry, K. M. Vranizan, and P. D. Wood. (1987) Relationships of plasma estradiol, testosterone, and sex hormone-binding globulin with lipoproteins, apolipoproteins, and high density lipoprotein subfractions in men. J. Clin. Endocrinol. Metab. **64**: 723-729

Stevenson, J. C., D. Crook, and I. F. Godsland. (1993) Influence of age and menopause on serum lipids and lipoproteins in healthy women. Atherosclerosis, **98:** 83-90

Stubbe, I., P. Hansson, A. Gustafson, and P. Neilsson-Ehle. (1983) Plasma lipoproteins and lipolytic enzyme activities during endurance training in sedentary men: Changes in high-density lipoprotein subfractions and composition. Metabolism, **32:** 1120-1128

Stunkard, A. J., and K. D. Brownell. (1981) Differential changes in plasma high density lipoprotein-cholesterol levels in obese men and women during weight reduction. Arch. Intern. Med. **141:** 1142-1146

Swinkels, L. M. J. W., H. J. C. van Hook, H. A. Ross, A. G. H. Smals, and Th. J. Benraad. (1991) Concentrations of salivary testosterone and plasma total, non-sex hormone-binding globulin-bound, and free testosterone in normal and hirsute women during administration of dexamethasone/synthetic corticotropin. Clin. Chem., **37(2):**180-185

Takegoshi, T, T. Haba, J. Hirai, C. Kitoh, T. Saga, Y. Yamazaki, and H. Mabuchi. (1990) Alterations of lipoprotein(a) in patients with diabetic nephropathy. Atherosclerosis, **83:** 99-100

Taskinen, M-R., and E. A. Nikkilä. (1981) High density lipoprotein subfractions in relation to lipoprotein lipase activity of tissues in man-- evidence for reciprocal regulation of HDL_2 and HDL_3 levels by lipoprotein lipase. Clin. Chim. Acta, **112:** 325-332

Terry, R. B., Stefanick, M. L., Haskell, W. L., and P. D. Wood. (1991) Contributions of regional adipose tissue depots to plasma lipoprotein concentrations in overweight men and women: possible protective effect of thigh fat. Metabolism **40 (7):** 733-740

Terry, R. B., P. D. Wood, W. L. Haskell, M. L. Stefanick, and R. M. Krauss. (1989) Regional adiposity patterns in relation to lipids, lipoprotein cholesterol, and lipoprotein subfraction mass in men. J. Clin. Endocrinol. Metab. **68(1):**191-199

Tietz[1], R. N. (1986) Textbook of Clinical Chemistry. W.B. Saunders,

Philadelphia.

Tietz[2], R. N. (1986) Fundamentals of Clinical Chemistry. W.B. Saunders, Philadelphia.

Thompson, G. R. (1990) A Handbook of Hyperlipidaemia. Current Science Ltd., London.

Utermann, G., F. Hoppichler, H. Dieplinger, M. Seed, G. Thompson and E. Boerwinkle. (1989) Defects in the LDL receptor gene affect Lp(a) lipoprotein levels: Multiplicative interaction of two gene loci associated with premature atherosclerosis. Proc. Natl. Acad. Sci. USA. **86:** 4171-4174

Väisänen, S., J. Gavert, A. Julkunen, E. Voutilainen, and I Penttila. (1992) Contents of apolipoprotein A-I, A-II and B of the human serum fractions for high-density and low-density lipoproteins prepared by common precipitation methods. Scan. J. Clin. Lab. Invest. **52(8):** 853-862

Van Aswegan, C. H., P. Hurter, C. A. van der Merwe, D. J. du Plessis. (1989) The relationship between total urinary testosterone and renal calculi. Urol. Res. **17(3):**181-183

Välimäki, M., K. Laitinen, R. Ylikahri, C. Ehnholm, M. Jauhiainen, J. M. Bard, J. C. Fruchart and M.-R. Taskinen (1991) The effect of moderate alcohol intake on serum apolipoprotein A-I-containing lipoproteins and lipoprotein (a) metabolism. Metabolism **40 (11):** 1168-1172

Warnik, G. R., J. Benderson and J. J. Albers. (1982) Dextran sulfate-Mg2+ precipitation procedure for quantitation of high density-lipoprotein cholesterol. Clin. Chem. **28(6):**1379-1388

Webb, A. T., D. A. Reaveley, M. O'Donnel, B. O'Connor, M. Seed, and E. A. Brown. (1993) Does cyclosporin increase lipoprotein(a) concentrations in renal transplant recipients? Lancet, **341:** 268-270

Weise, W. J., Y. Natori, J. S. Levine, Y. M. O'Meara, A. W. Minto, E. C. Manning, D. J. Goldstein, D. R. Abrahamson, and D. J. Salant. (1993) Fish oil has protective and therapeutic effects on proteinuria in passive Heymann nephritis. Kidney Int. **43:** 359-368

Witztum, J. and G. Schonfeld. (1979) High density lipoproteins. Diabetes, **28:** 326-336

Wong, Y. F., K. Mao, N. S. Panesar, E. P. L. Loong, A. M. Z. Chang, and Z. J. Mi. (1990) Salivary estradiol and progesterone during the normal ovulatory menstrual cycle in chinese women. Euro. J. Obstet. Gyneco. Reprod. Biol., **34:**129-135

Wood, P. D., and W. L. Haskell. (1979) The effect of exercise on plasma high density lipoproteins. Lipids, **14 (4)**: 417-427

Zumoff, B., G. W. Strain, J. Kream, J.O'Connor, R. S. Rosenfeld, J. Levin, and

D. K. Fukushima. (1982) Age variation of the 24-hour mean plasma concentration of androgens, estrogens, and gonadotropins in normal adult men. J. Clin. Endocrinol. Metab. **54:**534-538

Zumoff, B., R. S. Rosenfeld, M.. Friedman, S. O. Byers, R. H., Rosenman, and L. Hellman, (1984) Elevated daytime urinary excretion of testosterone glucuronide in men with the type A behavior pattern. Psychosom-Med. **46(3):**223-225

Zumoff, B., G. W. Strain, L. K. Miller, W. Rosner, R. Senie, D. S. Seres, and R. S. Rosenfeld. (1990) Plasma free and non-sex hormone-binding globulin-bound testosterone are decreased in obese men in proportion to their degree of obesity. J. Clin. Endocrinol. Metab. **71:** 929-931

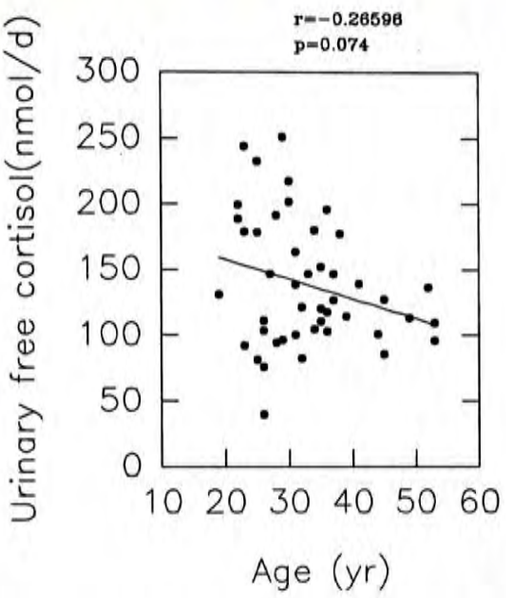
Yen, S.S.C. (1986) Chronic anovulation caused by peripheral endocrine disorders. In "Reproductive Endocrinology" 2nd ed. 1986, Yen, S.S.C. and Jaffe, R.B. (eds.), W. B. Saunders Co., Philadelphia.

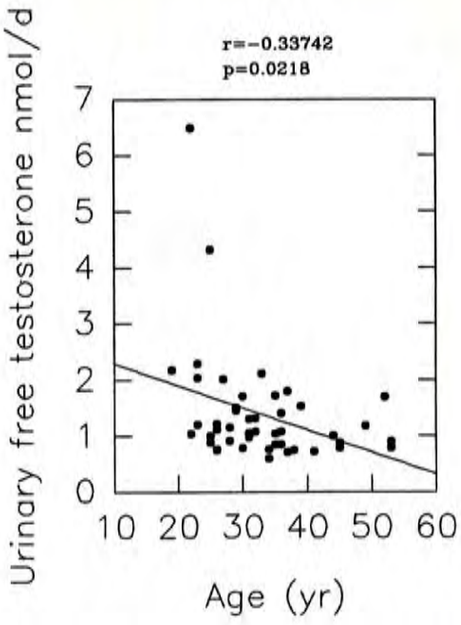
Appendices

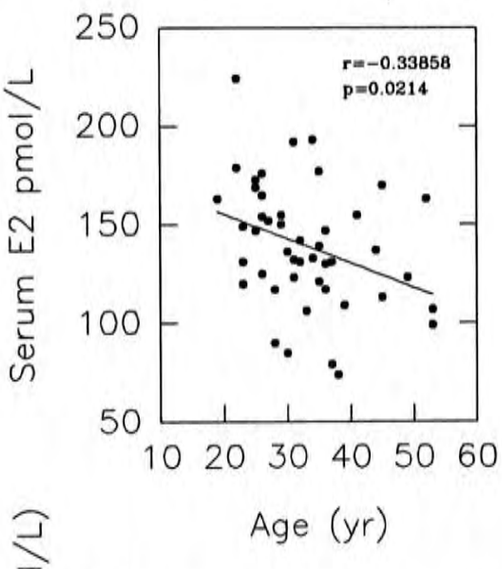
Appendix i: Parameter listing for cholesterol and triglyceride assayed by Cobas Bio automatic analyser

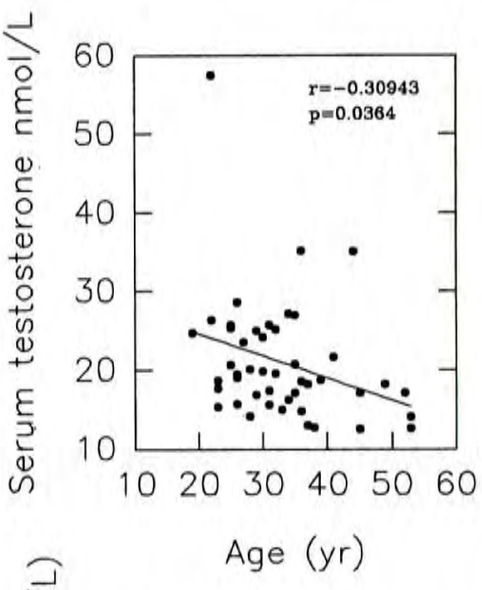
1. Units	mmol/L
2. Calculation factor	0
3. Standard 1 concentration	8.68 (for cholesterol), 3.26 for trig. 0.54 for HDL
4. Standard 2 concentration	4.34 (for cholesterol), 1.63 for trig. 1.08 for HDL
5. Stamdard 3 concentration	0 (for cholesterol and triglyceride), 2.17 for HDL
6. Limit	18
7. Temperature (Degree. C)	37
8. Type of analysis	5
9. Wavelength (NM)	520
10. Sample volume	5 µl (for cholesterol and trig.), 20 µl for HDL
11. Diluent volume	40 µl (for cholesterol and trig.), 60 µl for HDL
12. Reagent volume	300 µl
13. Incubation time	0
14. Start reagent	0
15. Time of first reading	5 second
16. Time interval	300 second
17. Nnumber of readings	2
18. Blanking mode	1
19. Printout mode	1

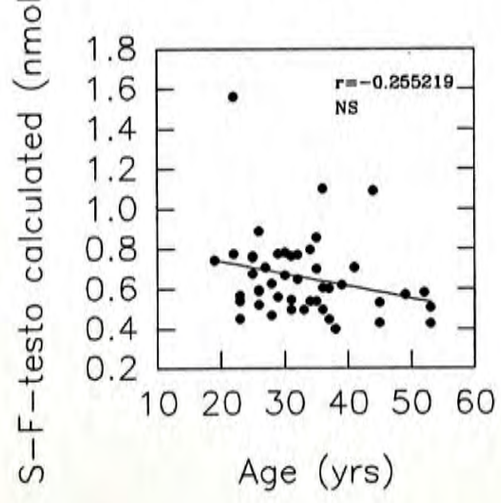
Appendix ii. a.--Correlation of age and sex hormone in serum and urine

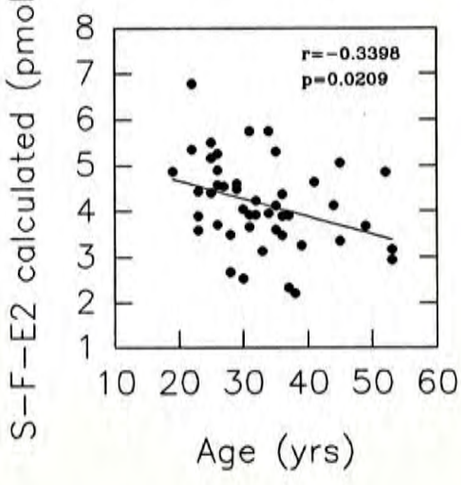




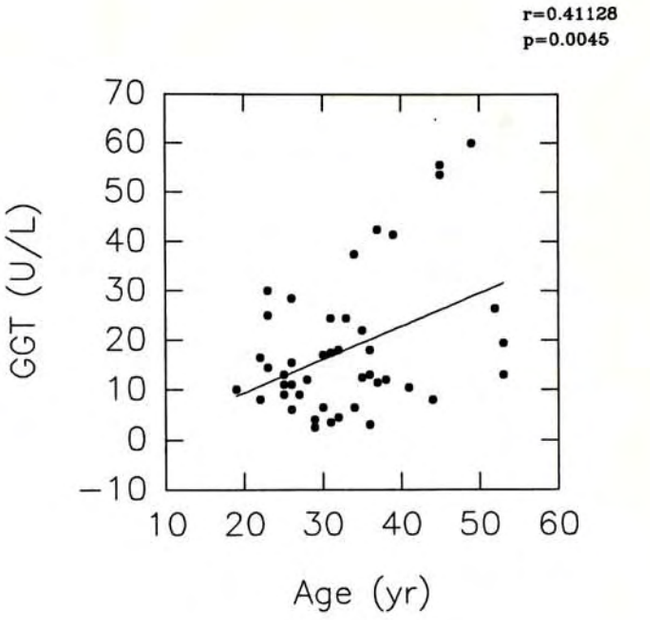


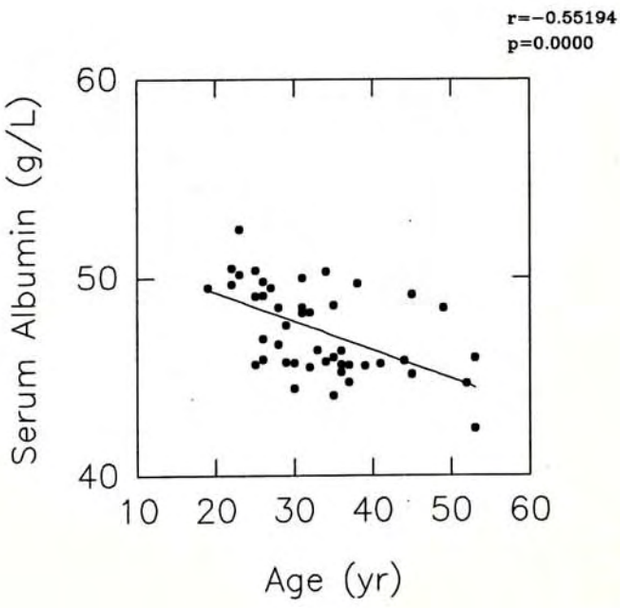




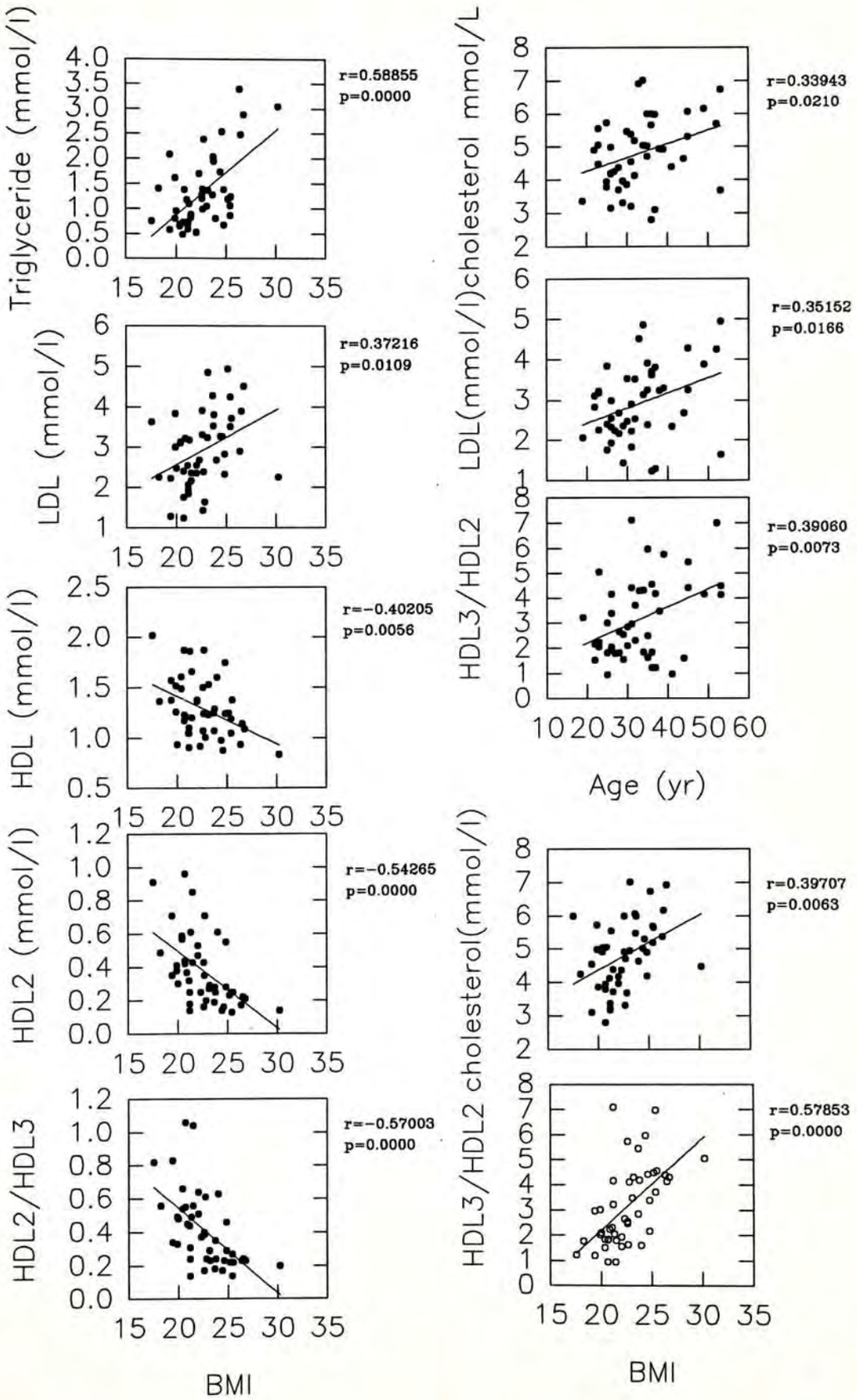


Appendix ii. b.--Association of age and serum albumin and γGT

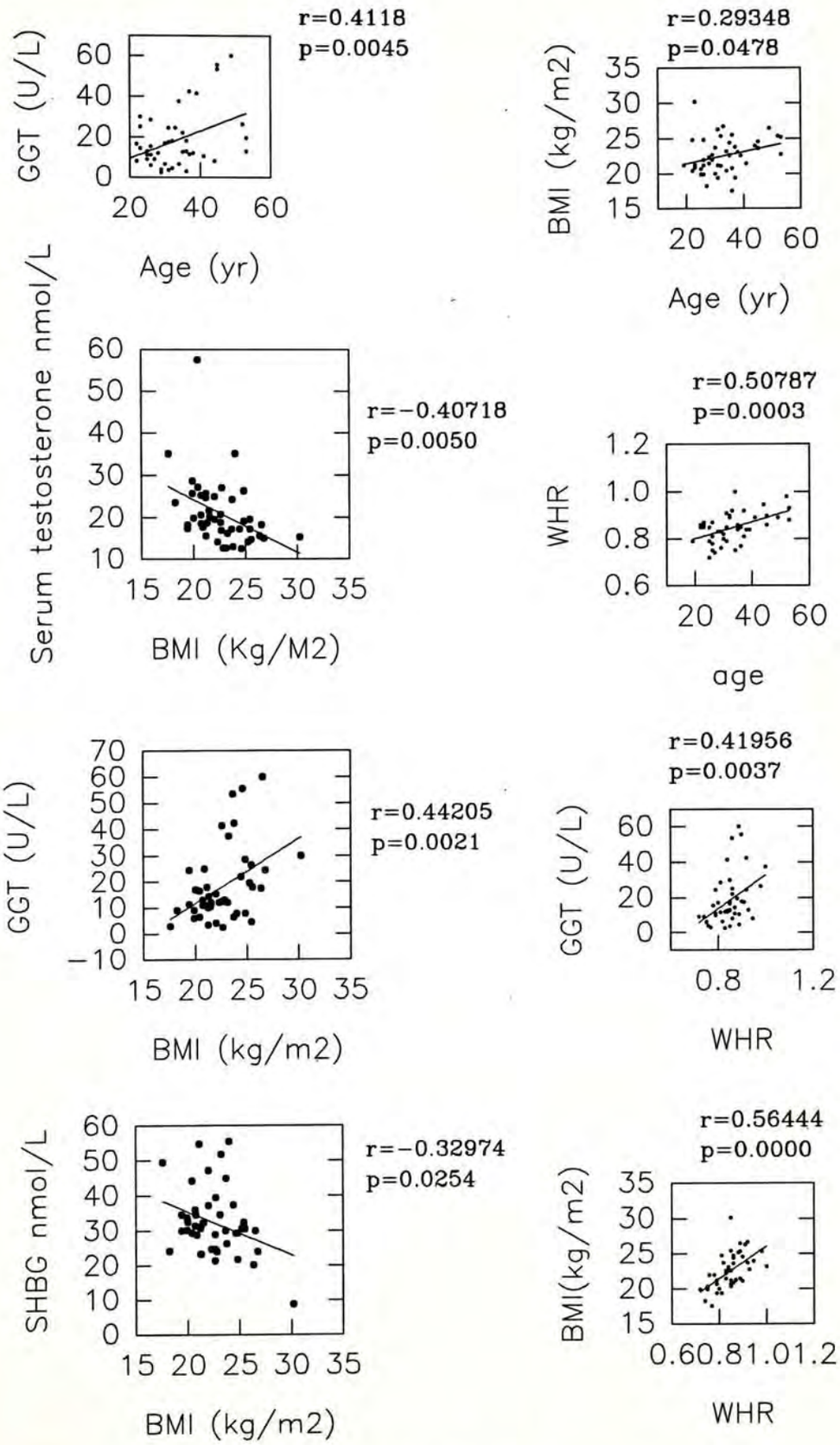




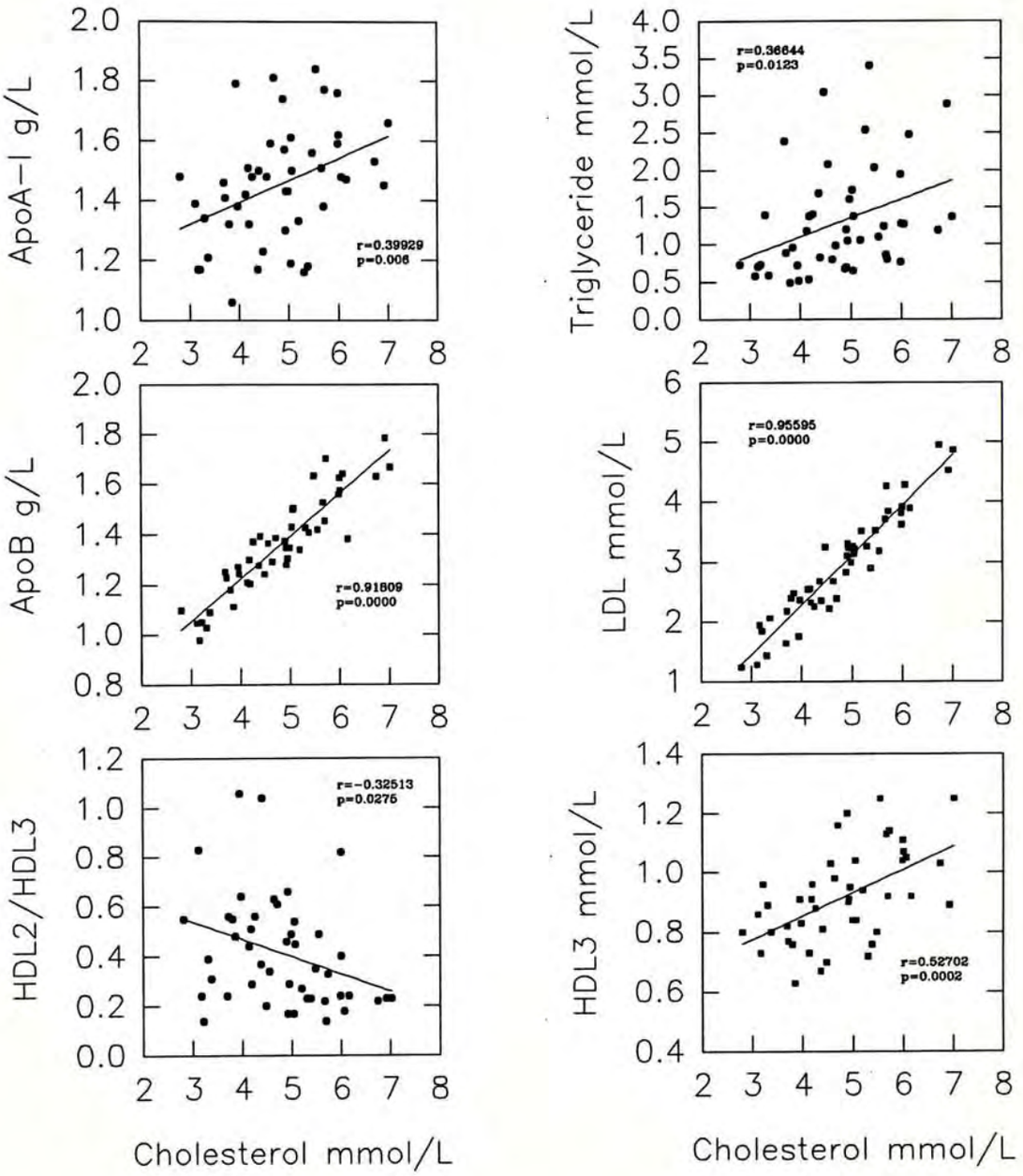
Appendix ii. c.-- Correlation of age, BMI and serum lipoprotein-lipids



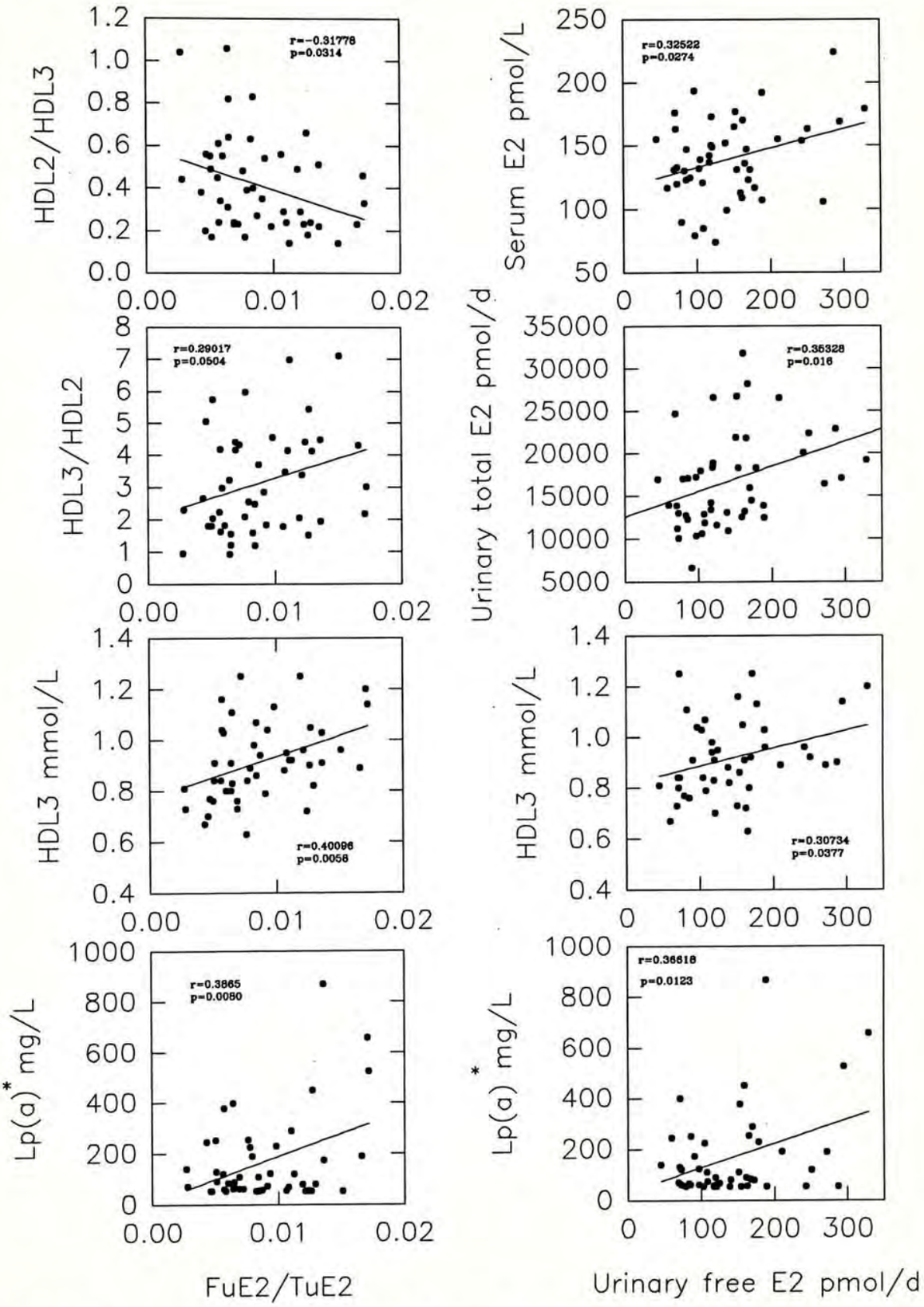
Appendix ii. d.-- Association of Age, BMI, WHR, GGT, SHBG, and serum testosterone.



Appendix ii. e.--Correlation of serum cholesterol and lipoprotein-lipids and apolipoprotein.

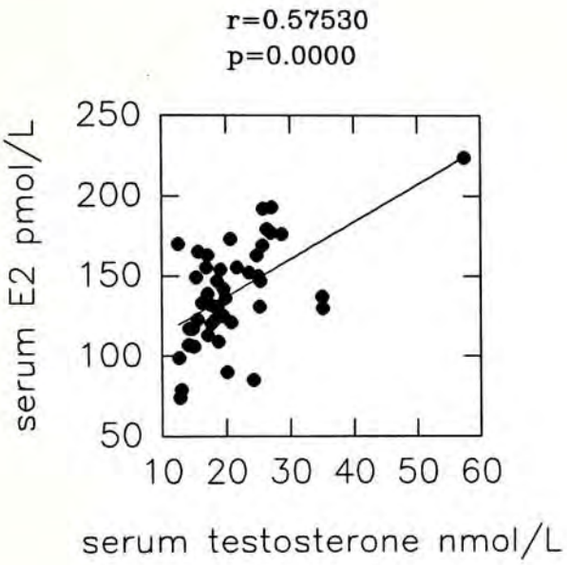


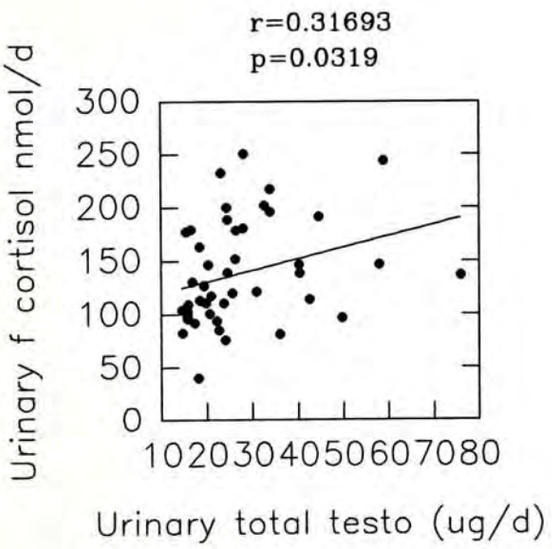
Appendix ii. f.--Association of urinary non-conjugated E_2 to lipoprotein-lipids, serum E_2 and urinary total E_2

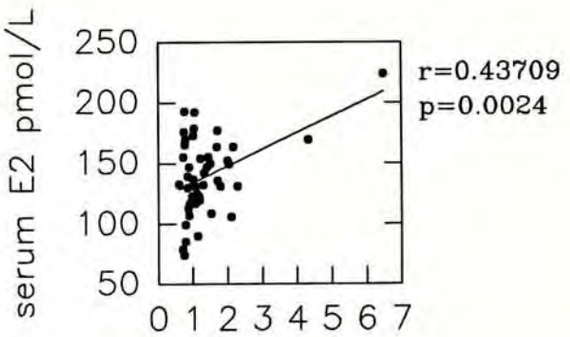


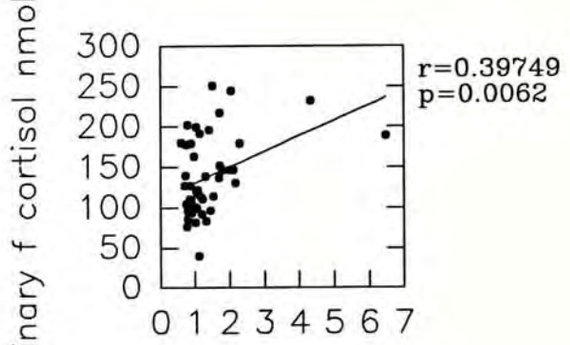
* For untransformed data only

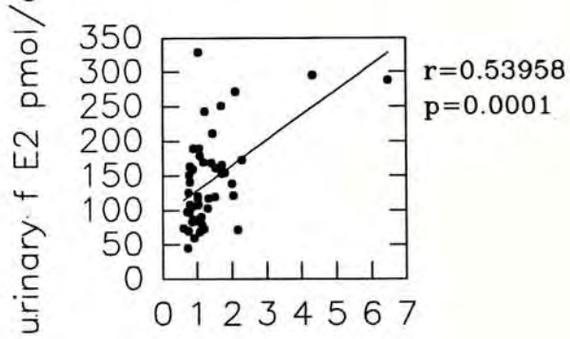
Appendix ii.g.--Correlation of urinary total & non-conjugated testosterone and sex hormone in serum and urine and urinary free cortisol

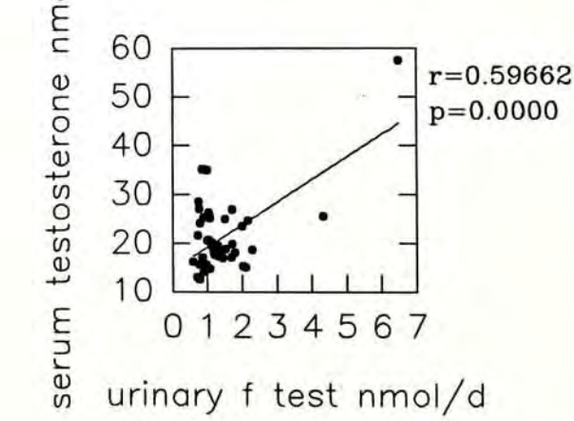




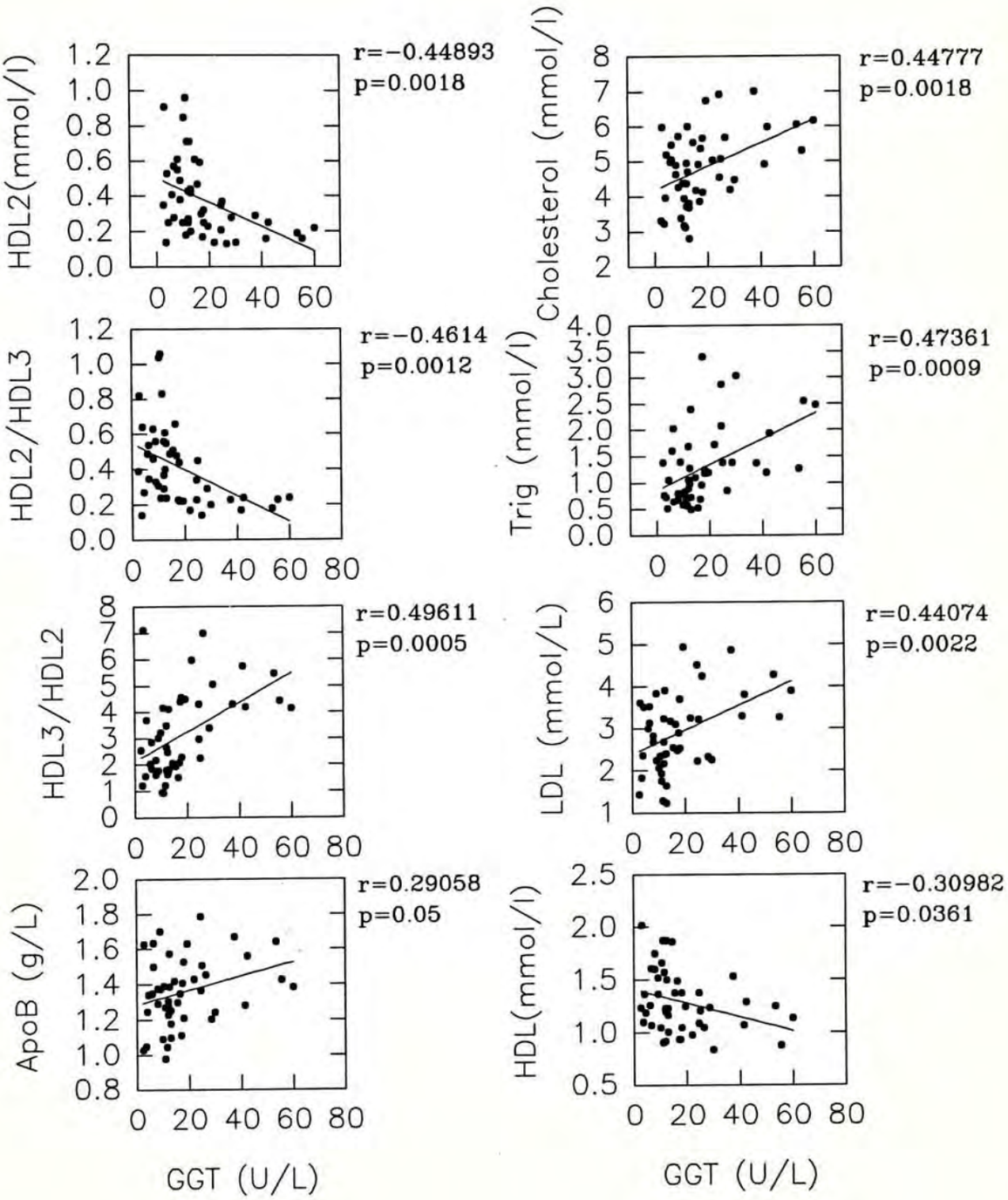




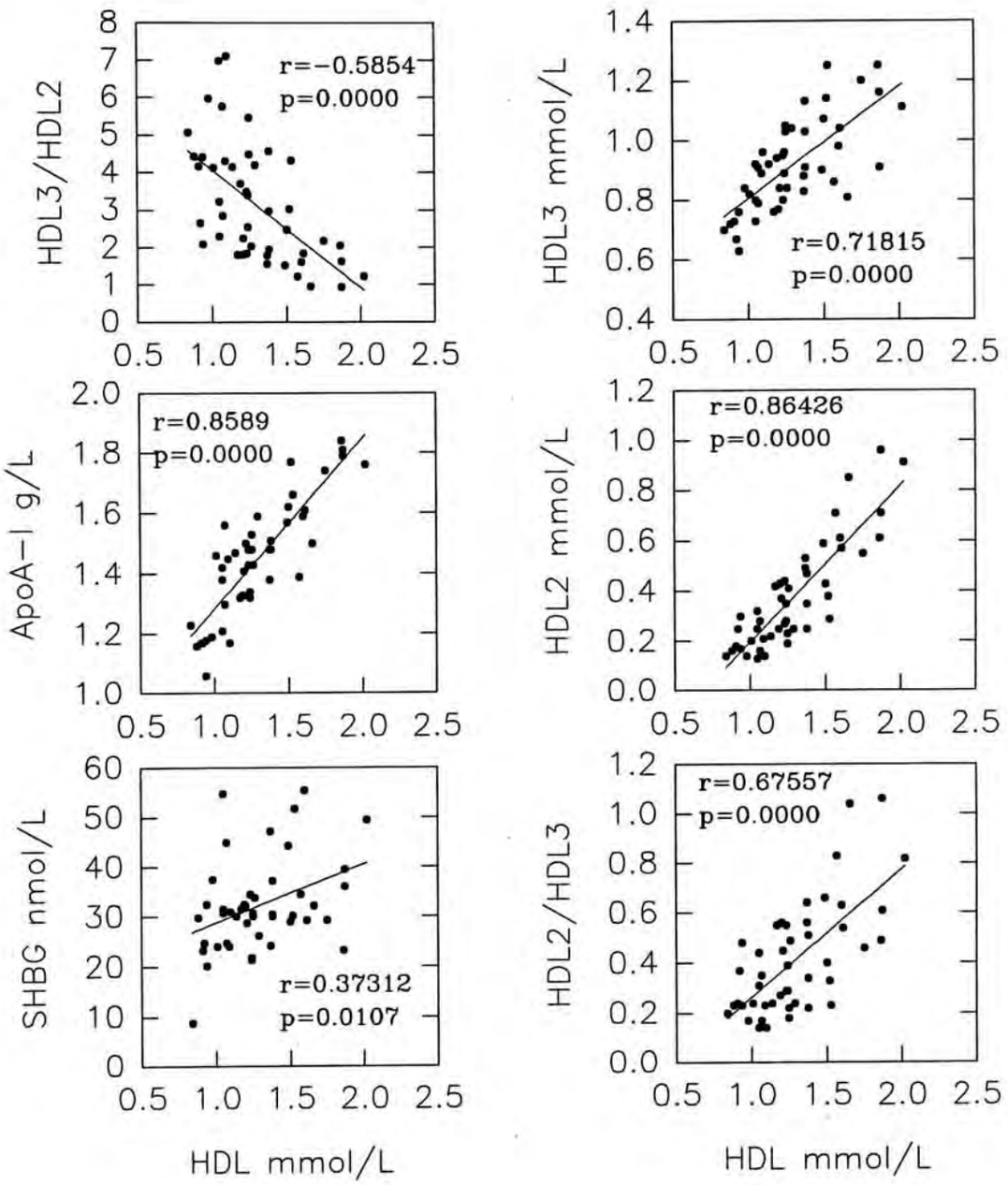




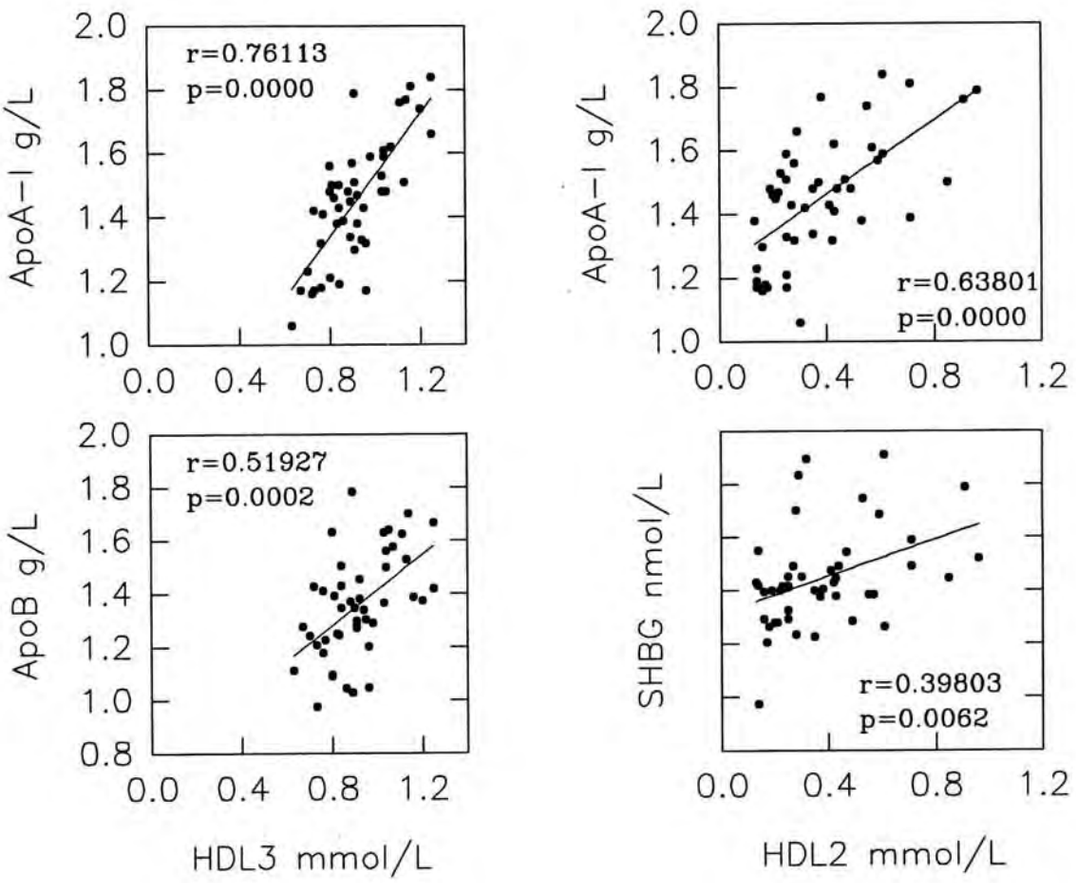
Appendix ii. h.--Correlation of serum γGT and lipoprotein-lipids and apolipoprotein



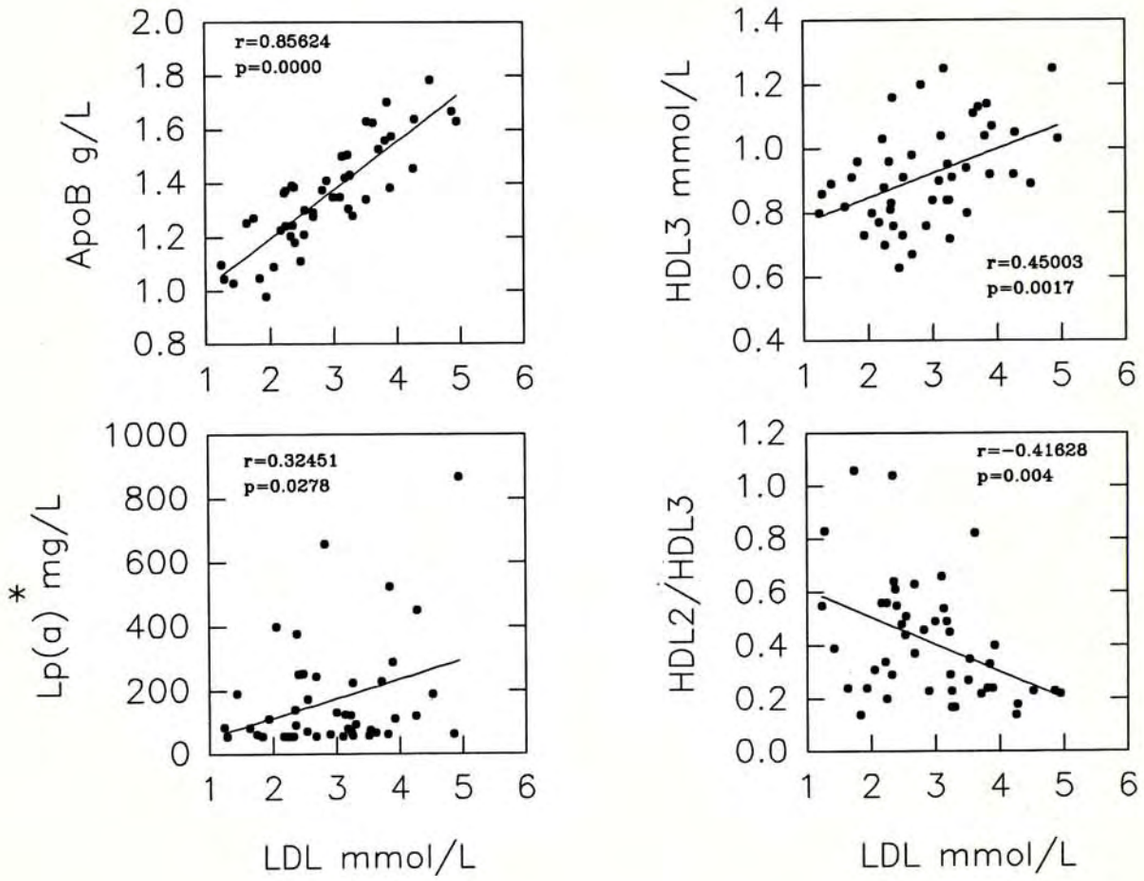
Appendix ii. i.--Correlation of HDL-C and other lipoprotein-lipids and SHBG



Appendix ii. j.--Correlation of HDL subfractions and apolipoproteins and SHBG

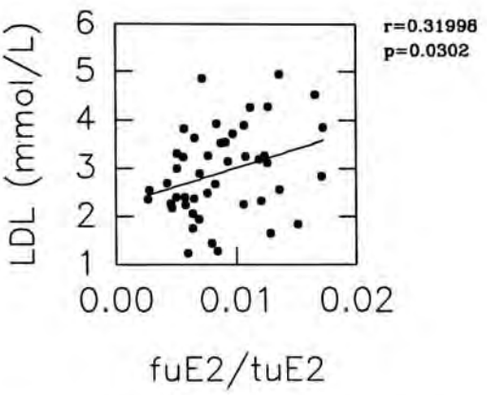


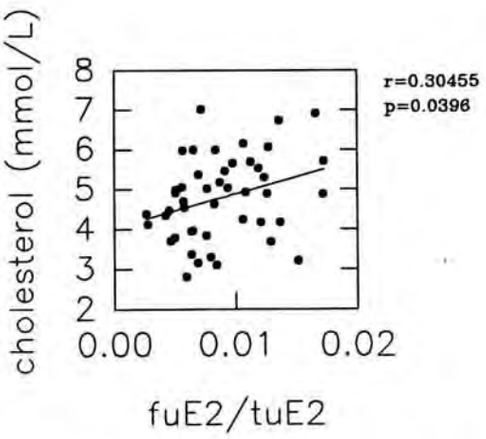
Appendix ii. k.--Correlation of LDL-C and HDL subfractions , Lp(a) and apoB

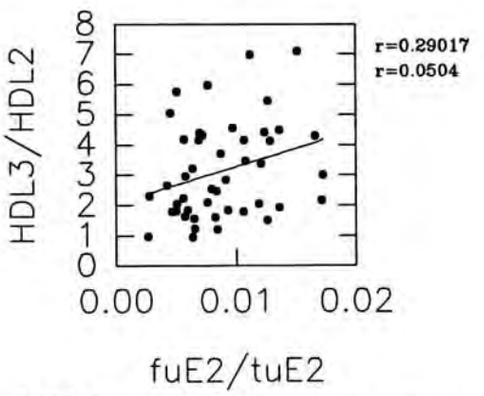


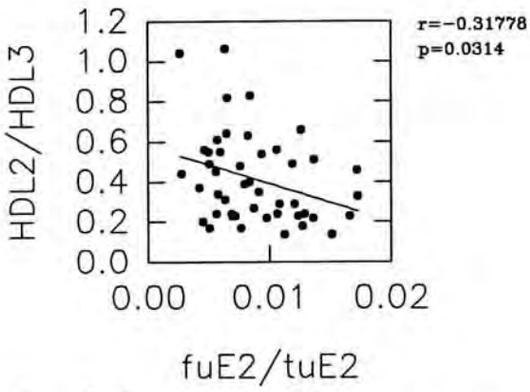
* untransformed data

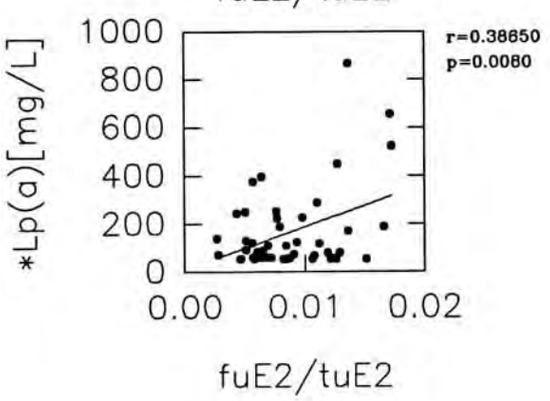
Appendix ii. l.--Correlation of the ratio of urinary free & urinary total estradiol* (fuE2/tuE2) *and lipoprotein-lipids

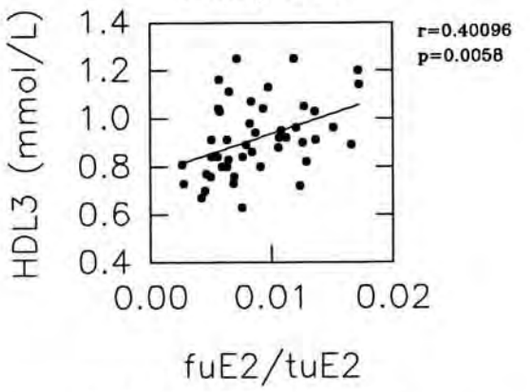


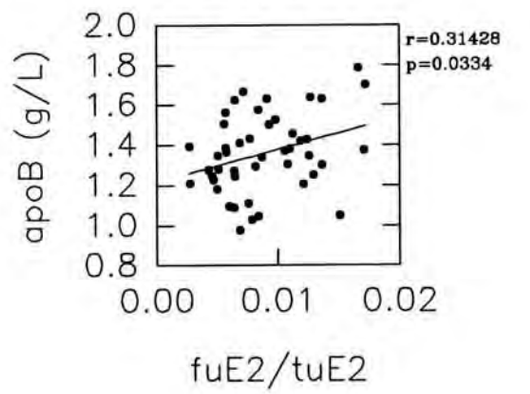






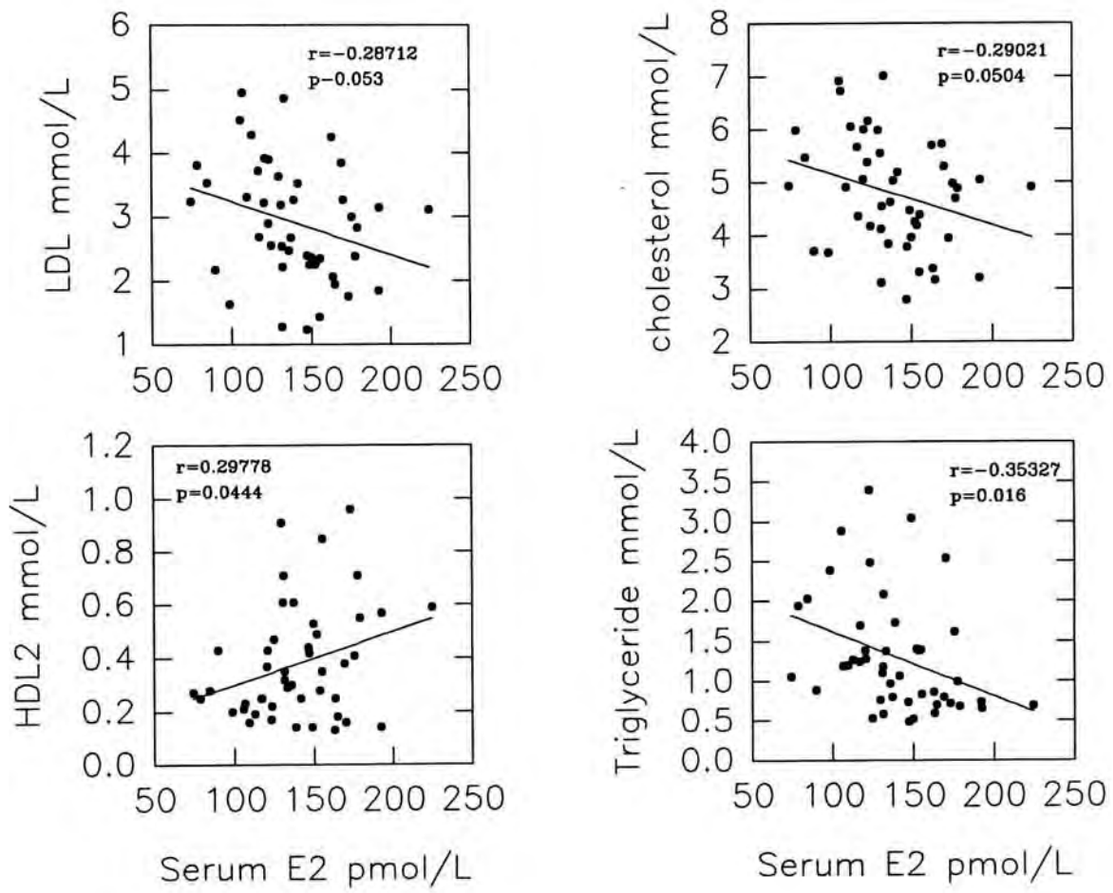




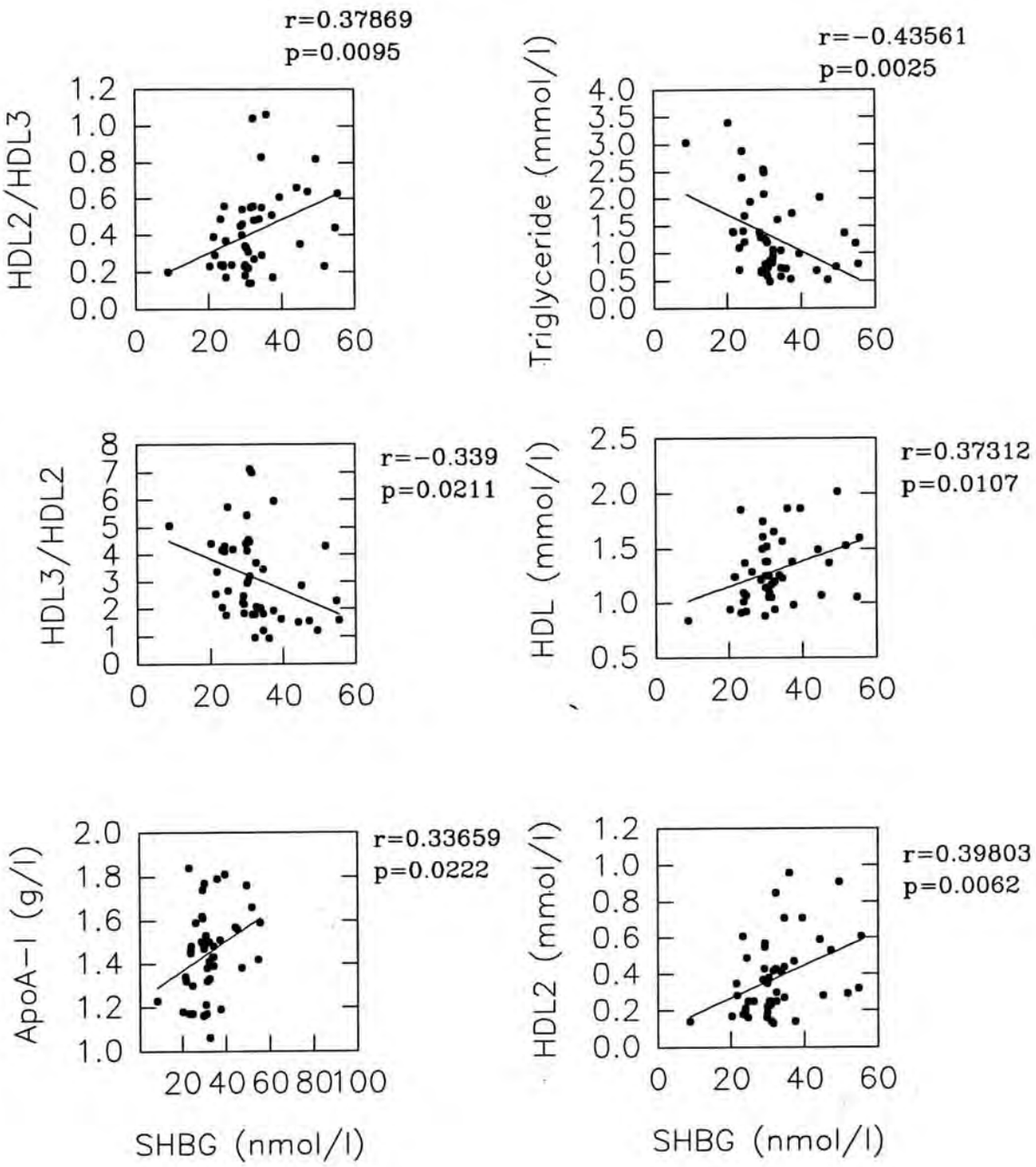


* untransformed data

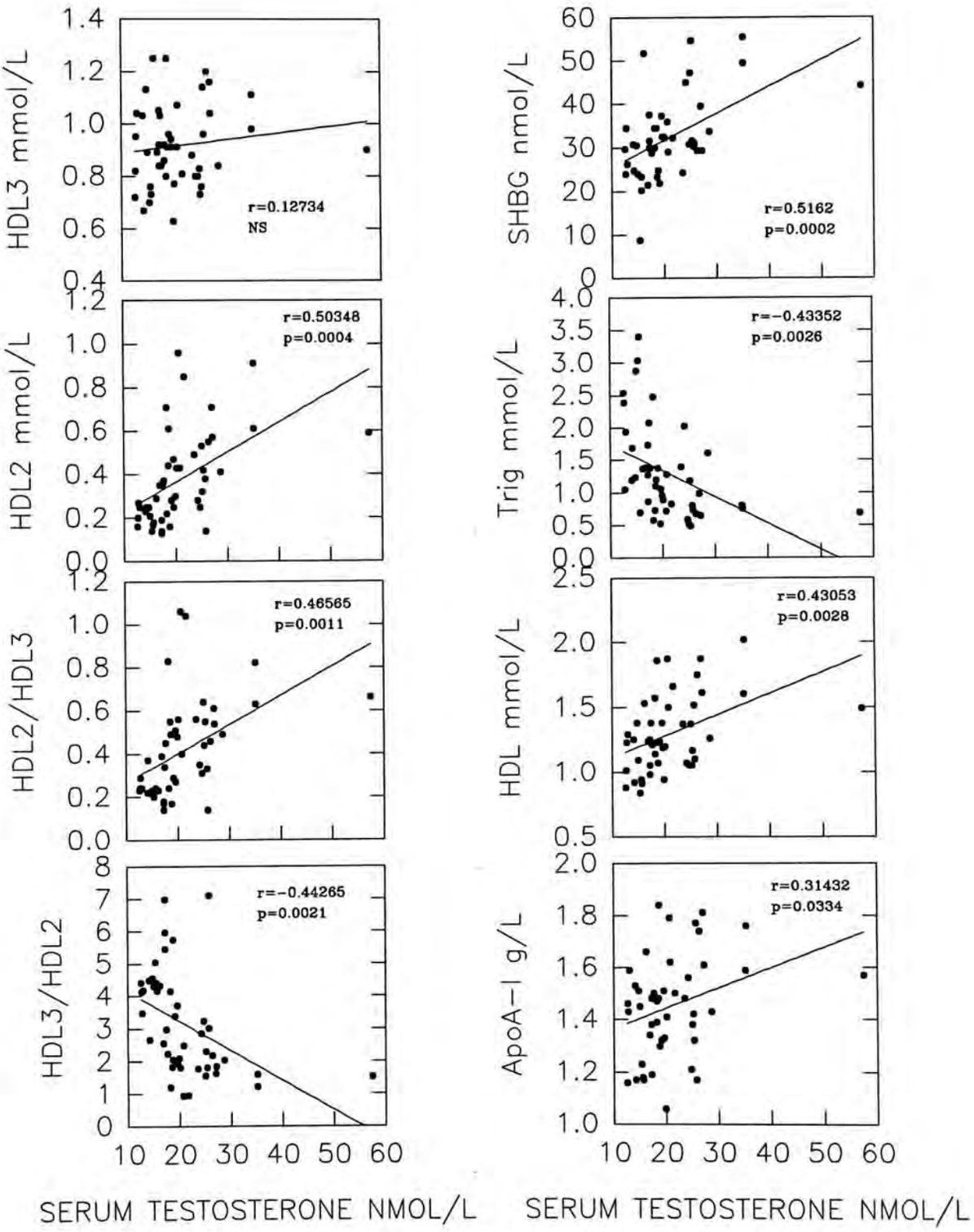
Appendix ii. m.--Correlation of serum estradiol and lipoprotein-lipids



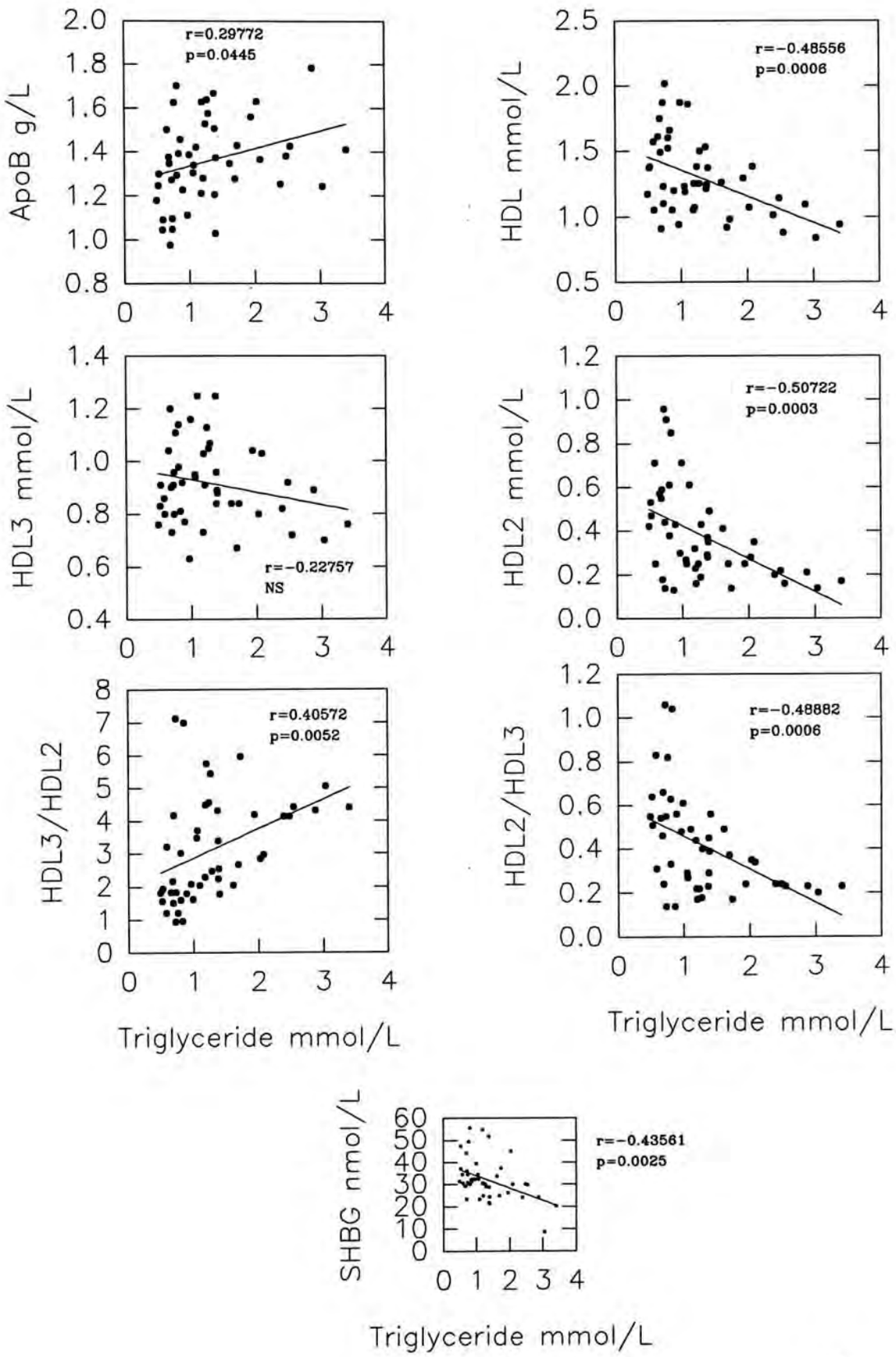
Appendix ii. n.--Correlation of SHBG and lipoprotein-lipids and apolipoprotein



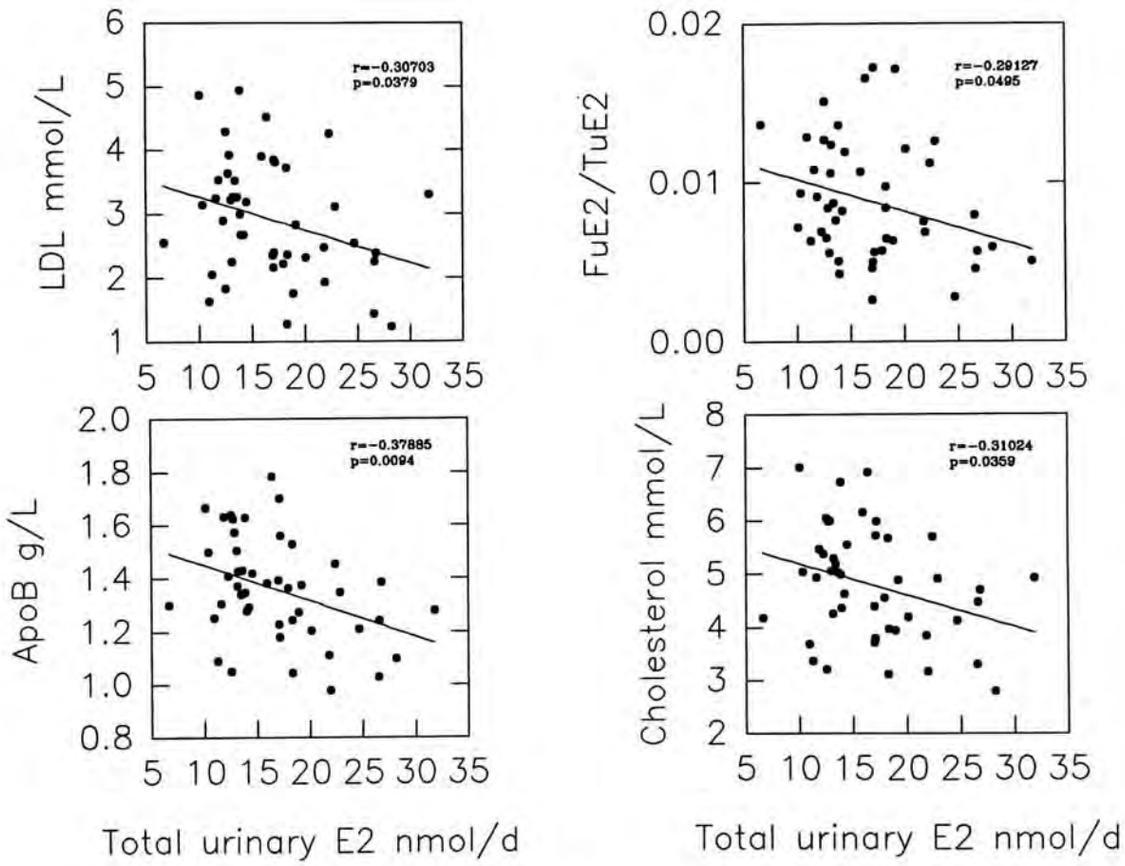
Appendix ii. o.--Correlation of serum total testosterone and lipoprotein-lipids, apolipoprotein and SHBG



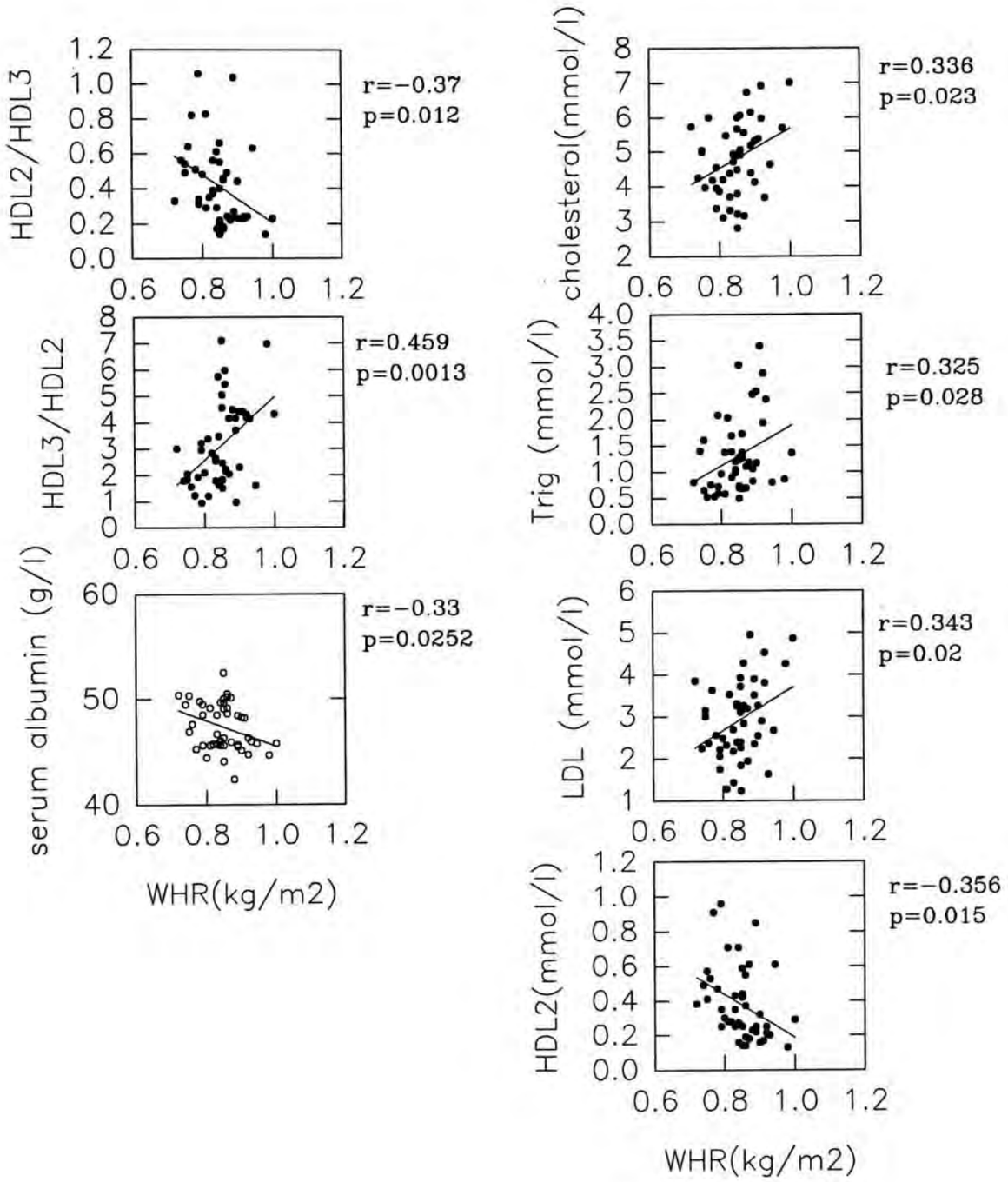
Appendix ii. p.--Correlation of triglyceride and lipoprotein-lipids and apolipoprotein



Appendix ii. q.--Correlation of urinary total estradiol and lipoprotein-lipids and apoB



Appendix ii. r.--Correlation of WHR and lipoprotein-lipids, and serum albumin



Appendix iii. List of references for Table IV. 2.

1. Schöneshöfer, M., and B. Weber. (1983) Specific estimation of fifteen unconjugated, non-metabolized steroid hormones in human urine. J. Steroid Biochem. **18:** 65-73

1a. Stahl, F., G. Dörner, I. Poppe, W. Rohde, G. Knappe, B. Stober, and B. Raabe. (1974) Radioimmunological determinations of unconjugated testosterone in the plasma and urine. Endokrinologie, **64:**63-73

1b. Kield, J. M., C. M. Puah, and G. F. Joplin. (1977) Measurement of unconjugated testosterone, 5-α-dihydrotestosterone and œstradiol in human urine. Clin. Chim. Acta. **80:** 271-284

2. Dai, W., S. Kuller, L. H. LaPorte, J. P. Gutai, L. Falvo-Gerard, and A. Caggiula. (1981) The epidemiology of plasma testosterone levels in middle-aged men. Am. J. Epidemiology, **114:** 804-816

3. Tietz, N. W. (1983) Clinical Guide to Laboratory Tests. W. B. Saunders.

4. Mauvais-Jarvis, P., G. Charransol, and F. Bobas-Masson. (1973) Simultaneous determination of urinary androstenediol and testosterone as an evaluation of human androgenecity. J. Clin. Endocrinol. Metab. **36:** 452

5. Doberne, Y., and M. I. New. (1976) Urinary androstenediol and testosterone in adults. J. Clin. Endocrinol. Metab. **42:** 152

6. Speight, A. C., K. W., Hancock, R. E. Oakay. (1979) Non-protein bound estrogens in plasma and urinary excretion of unconjugated estrogens in men. Clin. Endocrinol. (Oxf) **10 (4):** 329-341.

7. Ikuko, K. et al., (1992) Determinants of sex hormone levels in men as useful indices in hormone-related disorders. J. Clin. Epidemiol. **45 (12):** 1417-1421

8. Auletta, F. J., B. V. Caldwell, and G. L. Hamilton. (1979) Androgens: testosterone and dihydrotestosterone. In "Methods of Hormone Radioimmunoassay" 2nd ed., Jaffe, B. M. and H. R. Behrman (eds.) Academic Press, U.S.A.

9. DPC coat a count Estradiol, DPC, U.S.A.

10. Shanghai Institute of Endocrinology.